Long-chain fatty acid incorporation into, and long-chain alcohol production by, yeasts

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LONG-CHAIN FATTY ACID INCORPORATION INTO, AND LONG-CHAIN ALCOHOL PRODUCTION BY, YEASTS

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SUMMARY

Anaerobically grown Saccharomyces cerevisiae Y185, grown in the presence of 30 mg oleic acid l^{-1}, harvested from mid exponential-phase cultures incorporated approximately 50% of the exogenously provided oleic acid supplement. Fractionation of spheroplast lysates using discontinuous sucrose-density gradients and subsequent identification of subcellular organelles revealed that the oleic acid supplement was predominantly incorporated into low-density vesicles (0.25 to 0.50 μm diam.) and plasma membranes. Pulse-labelled experiments with [1-^{14}C]oleic acid provided some evidence for the exchange of lipids between low-density vesicles and the plasma membrane.

Lipid extracts from whole organisms of anaerobically grown Sacch. cerevisiae Y185 contained a high proportion of free oleic acid. When organisms were converted to spheroplasts over a period of 1 h there was a transfer of free oleic acid to triacylglycerol and sterol-ester fractions, possibly induced by the altered environmental conditions. Isolated plasma membranes contained a high proportion of incorporated oleic acid associated with triacylglycerols and, to a lesser extent, sterol esters. Analysis of lipid fractions isolated from low-density vesicles showed a very high proportion of oleoyl residues associated with triacylglycerols and a smaller proportion associated with sterol esters. The data reported provide further evidence for a role for intracellular low-density vesicles in envelope growth in Sacch. cerevisiae.

Fourteen yeast strains from six genera were analysed for the presence of long-chain alcohols. Six strains from three genera contained long-chain alcohols, highest levels being found in Candida albicans. The alcohols were identified and determined by TLC, GLC and GLC-mass spectrometry. The major long-chain alcohols synthesized by these organisms were saturated primary alcohols with C_{14}, C_{16} or C_{18} chain length. Unsaturated long-chain alcohols were not detected.

In cultures of C. albicans, synthesis of long-chain alcohols occurred only after the end of exponential growth. The long-chain alcohols were predominantly present as free alcohols. In general, the content of hexadecanol was greatest in self-induced anaerobic cultures, while in aerobic cultures of C. albicans octadecanol predominated. Content of tetradecanol was always lowest. Contents of all three classes of alcohol increased as the concentration of glucose in aerobic cultures, harvested after 168 h incubation, was raised from 1.0 to 30.0% (w/v). However, in 168-h self-induced anaerobic cultures of C. albicans, peak contents in organisms were found using media containing 10% (w/v) glucose. Substituting glucose (10%, w/v), in aerobic cultures, by the same concentration of galactose greatly decreased contents of long-chain alcohols, while 10% (w/v) glycerol virtually abolished their synthesis.

Fatty-acyl chain lengths of the major lipid classes isolated from organisms grown under aerobic and self-induced anaerobic conditions reflected those of the long-chain alcohols produced by C. albicans. Supplementing self-induced anaerobic cultures with 10 mg odd chain-length fatty acids l^{-1} induced synthesis of odd

chain-length alcohols. Maximum conversion of odd chain-length fatty acid to the corresponding alcohol was observed with heptadecanoic acid. The effect of glucose on the production of heptadecanol from exogenously provided heptadecanoic acid was similar to that observed with the three major even chain-length alcohols under unsupplemented conditions. Cell-free extracts of C. albicans catalyzed in vitro conversion of palmitoyl-CoA to 1-hexadecanol.

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INTRODUCTION

The earliest investigations into fungal lipids date as far back as the early 1870s when it was discovered that the ergot fungus, Claviceps purpurea, contained 30% fat. Much of the work on fungal lipids in the following 50 years was devoted to screening organisms for potential industrial fat producers. During this time, it was recognized that fungi vary considerably in their ability to produce fat and, more importantly, that the degree of fat production varies according to environmental conditions. Research in the following half century emphasized defining culture conditions and nutritional factors favourable for fat production. Several species became known as 'fat-producing' fungi. These included some species of the filamentous fungi Aspergillus, Penicillium, Mucor, Fusarium, and the yeasts Lipomyces, Rhodotorula, and Candida. Some of the objectives of this type of research were to determine the commercial potential of fungal fat and, therefore, much of the work focused on efficiencies of carbon substrate utilization and the possibility of using relatively inexpensive waste products as substrates for growth.

In the early 1950s there was a transition from an era of research into fat production to a period of lipid analysis, mainly due to the development of chromatographic techniques, particularly thin-layer and gas-liquid chromatography. It was only since these developments that reliable data on the lipid composition of organisms have been reported. While it was recognized that triacylglycerols were the predominant lipids that accumulated in yeasts

and other fungi, the complex nature of lipid extracts became more apparent. As analytical techniques became more refined, individual sterols, phospholipids and other lipids were identified which led to the abandonment of the old view that the only function of importance for fats was their role as long-term storage materials. The availability of radio-isotopes suitable for tracer studies in the late 1940s, combined with chromatographic techniques, made possible the next major advance in lipid research. This was elucidation of biochemical pathways and reaction mechanisms, as well as studies on enzyme biochemistry.

Since the early 1970s, there has been an escalation in interest in yeast lipids. Researchers, in the past, had restricted themselves to establishing the nature of the lipases and the biosynthetic pathways for fatty acids, phospholipids and sterols mainly in strains of Saccharomyces cerevisiae, rather than trying to elucidate the main mechanisms by which numerous other yeasts could accumulate large quantities of lipids other than those associated with cellular membranes. Progress has also been stimulated by the interest of the petroleum industry in using micro-organisms to synthesize other lipids from hydrocarbons or to produce other valuable products during growth on these compounds (Higgins and Gilbert, 1978). Production of single-cell protein and degradation of crude oils are the two principal reasons for current interests in microbial utilization of aliphatic hydrocarbons (Ratledge, 1978).

With the increasing demands for fats and oils for edible and industrial purposes, many assessments have been made to find new

lipid sources other than conventional plant and animal sources. As a result, lipids from microbial origins were found to be a possible route. Microbes offer the advantages of ready availability of monocultures, control of environmental parameters and relatively simple exploitation of genetic mutants (Rattray, 1984; Yoon and Rhee, 1983). Amongst micro-organisms, yeasts and other fungi generally give highest lipid contents (Hunter and Rose, 1971; Ratledge, 1980). Few bacteria have a natural propensity to accumulate lipids though some species of Mycobacterium, Nocardia and Corynebacterium contain large amounts of lipid in their cell envelopes (Ratledge, 1980). These bacteria sometimes produce toxic or antigenic compounds and, therefore, their subsequent use on a large scale would have to be approached rather cautiously.

YEAST LIPIDS

Total Lipid Content

The lipid content of numerous yeast strains has been reported (Hunter and Rose, 1971; Ratledge and Evans, 1987; Weete, 1980). In general, it is possible to distinguish two distinct groups of yeasts. There are those species that contain up to 18-20% of the cell dry weight as lipid, with a majority possessing between 7 and 15%. Secondly, there is a smaller class of yeasts, sometimes referred to as the 'fat yeasts' but now more accurately classified as 'oleaginous yeasts' (Thorpe and Ratledge, 1972), which have total lipid contents ranging from 25-80% of the cell dry weight. However, it must be emphasized that this is only an arbitrary division since several oleaginous yeasts can easily be grown to

have lipid contents of less than 10% and, in general, lipid production can be manipulated by varying culture conditions (Ratledge and Evans, 1987; Weete, 1980).

Several techniques have been employed to extract lipids from yeast cells, and it is generally accepted that efficient extraction of lipids relies on disruption of the cell wall (Hunter and Rose, 1971; Nyns et al., 1968; Sobus and Holmlund, 1976), followed by removal of lipids using various solvents. With regard to maximum extraction of total cellular lipid, the method of disruption and solvent mixture used should be optimized for the particular yeast under investigation (Naganuma et al., 1982; Sobus and Holmlund, 1976; Suzuki et al., 1973). It must be pointed out that the vast majority of analyses reported on the total lipid content of yeasts are only crude estimations. Ratledge and Evans (1987) maintain there are three main pitfalls in estimating total yeast lipid, these being (1) incomplete extraction of lipid, (2) co-extraction of non-lipid material, and (3) lipolysis of the lipid during extraction. These pitfalls, combined with the fact that total lipid evaluations are often obtained gravimetrically which requires relatively large amounts of biomass and also relies on the accuracy of weighing milligram quantities of extract, indicate that the possibility of under- or over-estimations should always be considered.

Lipid Composition

Lipid extracts can be easily separated into neutral and polar lipid fractions using chromatographic techniques such as thin-layer

chromatography and column chromatography. Common neutral lipids in yeasts are triacylglycerols and sterol esters, while the predominant polar lipids are phospholipids but may also include some sphingolipids and glycolipids. Individual lipid classes in yeasts, and even components such as fatty-acyl residues within a particular lipid class, will vary in proportions according to culture age and conditions under which the organism is grown (Cottrell et al., 1981; Hossack et al., 1977; 1979; Ratledge and Evans, 1987; Weete, 1980).

Fatty acids. Although free fatty acids do not constitute a major lipid class in yeasts, fatty-acyl residues are very important as components of many lipids. Significant amounts of free fatty acid (approximately 5% of total lipid) have, however, been reported in Sacch. cerevisiae (Castelli et al., 1969; Hunter and Rose, 1972), Lipomyces starkeyi (Suzuki and Hasegawa, 1974a), Candida tropicalis (Greshnykh et al., 1968) and various other members of the genus Candida (Thorpe and Ratledge, 1972), but are now considered to be largely artefacts produced during extraction in which inadequate precautions have been taken to prevent action of lipases and phospholipases (Ratledge, 1982; Ratledge and Evans, 1987).

In general, the fatty-acyl composition of yeast lipids shows a preponderance of straight-chain C_{16} and C_{18} residues which can be saturated, mono-unsaturated or polyunsaturated (Hunter and Rose, 1971; Ratledge and Evans, 1987). Yeast lipids generally contain appreciable proportions of unsaturated fatty-acyl residues, oleic acid ($C_{18:1}$) being a major compound. Oleic acid (cis-octadec-9-enoic acid) has been determined to be the $C_{18:1}$ residue in

C. tropicalis (Mishina et al., 1973), L. starkeyi (Suzuki and Hasegawa, 1974b), and Mucor rouxii (Safe and Duncan, 1974). Only very low proportions of vaccenoyl residues (cis-octadec-11-enoic acid; Southwell-Kelly and Lynen, 1974) and traces of cyclopropane fatty-acyl residues (Kates and Paradis, 1973), both of which are major fatty-acyl components of many bacterial lipids (Johnston and Goldfine, 1982; Kates, 1970), have been detected in yeasts. Polyunsaturated fatty acids are usually associated with specific yeast strains (Johnson and Brown, 1972). In Sacch. cerevisiae, polyunsaturated fatty-acyl residues do not occur due to the absence of the appropriate Δ^{12} desaturase (Ratledge and Evans, 1987). In other yeasts, proportions of linoleoyl ($C_{18:2}$, Δ^{9}, Δ^{12}) and linolenoyl ($C_{18:3}$, Δ^{9}, Δ^{12}, Δ^{15}) residues can vary widely from 0.2 to 53.9% and less than 0.1 to 17.5% of total fatty-acyl residues respectively (Cottrell et al., 1986; Kaneko et al., 1976; Viljoen et al., 1986). In general, these fatty-acyl residues tend to be associated more with phospholipids than with triacylglycerols.

Cottrell et al. (1986) in an examination of 18 strains of Sacch. cerevisiae and its synonyms, all grown in glucose-yeast nitrogen base medium, observed changes in the proportion of palmitoyl residues ($C_{16:0}$) from 4.4 to 16.8%, of palmitoleoyl residues ($C_{16:1}$) from 33.7 to 37.1%, of stearoyl residues ($C_{18:0}$) from 1.6 to 11.5%, and of oleoyl residues ($C_{18:1}$) from 28.2 to 45.3%. Despite the variations in fatty-acyl composition of this and other yeasts, these workers have considered that these differences are sufficiently distinct that they could be used as a guide to the classification of yeasts (Kock et al., 1985; Viljoen et al., 1986).

Shorter chain-length fatty-acyl residues, lauroyl ($C_{12:0}$) and myristoyl ($C_{14:0}$) as well as the longer ones, arachidoyl ($C_{20:0}$), behenoyl ($C_{22:0}$) and erucoyl ($C_{22:1}$), tend only to appear occasionally as trace amounts. Welch and Burlingame (1973) found C_{20} to C_{30} fatty-acyl residues in Sacch. cerevisiae, but these only accounted for 1 to 2% of the total fatty-acyl component.

Odd chain-length fatty acids, which do not usually occur in yeasts, may constitute a major proportion of the yeast fatty-acyl residues when organisms have been grown on odd chain-length alkanes (Ratledge, 1980; Thorpe and Ratledge, 1972) or odd chain-length fatty acids (Bell, 1973), thus establishing that there is no intrinsic reason against these residues being able to fulfil a useful function for the organism. Several yeasts are also known to produce considerable quantitites of dicarboxylic acids after growth on alkanes (Hill et al., 1986; Shiio and Uchio, 1971) and various patents covering their production have been taken out (Fukui and Tanaka, 1981; Rehm and Reiff, 1981).

Acylglycerols. Mono-, di- and tri-acylglycerols, by convention (Hirschmann, 1960; IUPAC-IUB Commission on Biochemical Nomenclature, 1967) are designated acyl-sn-glycerols. Triacylglycerols are triesters of glycerol and fatty acids, and are the principal storage lipid in yeasts. Diacylglycerols and mono-acylglycerols, which are diesters and monoesters of glycerol and fatty acids respectively, have also been detected in lipid extracts from yeasts (Kates and Baxter, 1962). The diacylglycerols are mostly 1,2-diacyl-sn-glycerols, although they include small quantities of

1,3-diacyl-sn-glycerols. The mono-acylglycerols which are found in yeast extracts are probably 1-acyl-sn-glycerols.

Diacylglycerols occur as intermediates in the biosynthesis of some phospholipids (Taylor and Parks, 1979) and therefore would not be expected to be present in more than trace amounts. Mono-acylglycerols are not storage lipids or intermediates in the biosynthesis of any other lipid. The frequent reporting of both mono- and di-acylglycerols in yeast lipids can probably be attributed to lipolysis occurring, either through the action of lipases on triacylglycerols (Nurminen and Suomalainen, 1970) or phospholipases on phospholipids (Harrison and Trevelyan, 1963), during lipid extraction. For these reasons Ratledge (1980) and Ratledge and Evans (1987) feel that it is not unreasonable to assume that the initial triacylglycerol content of a yeast be taken as the sum of all the acylglycerol molecules found in the lipid extract.

Triacylglycerols differ in the nature and positional distribution of the fatty-acyl residues. In mammalian lipids, unsaturated fatty-acyl residues preferentially occupy the sn-2 position in the glycerol molecule, and this appears to hold true for yeast lipids (Meyer and Block, 1963). Suzuki and Hasegawa (1974 b,c), using an unselective method of determining the positional distribution of fatty-acyl groups in the triacylglycerols from L. starkeyi, revealed that the sn-2 position was predominantly occupied by the unsaturated fatty-acyl residues $C_{16:1}$, $C_{18:1}$ and $C_{18:2}$. The sn-1 and sn-3 positions were not distinguished in this work. Thorpe and Ratledge (1972) also showed a similar distribution of unsaturated fatty-acyl residues in the

triacylglycerols of Candida sp. 107 and C. tropicalis. However, when Candida 107 contained high proportions of shorter-chain fatty acids ($C_{14:0}$ and $C_{15:0}$), following growth on tetradecane or pentadecane, these fatty-acyl groups could be accommodated at the sn-2 position.

A more complete stereospecific analysis of yeast triacylglycerols by Haley and Jack (1974) showed that, in L. lipofer, the sn-1 position had a preponderance of unsaturated residues (mainly $C_{18:1}$) to approximately 85% of the total acyl groups, while the saturated residue, $C_{16:0}$, constituted only 14% of the residues here. However, $C_{16:0}$ and $C_{18:0}$ comprised 38% of the total substituents at the sn-3 position. In keeping with the observations of those mentioned above, there was virtually no saturated fatty-acyl groups at the sn-2 position. Further analysis of this yeast by Phornpiboonya and Jack (1980) revealed that as cultures aged, the greatest changes occurred at the sn-3 position and not at the other two.

Phospholipids. Phospholipids are the fatty-acyl diesters of sn-glycerol and the amount found in yeasts usually lies between 3 and 7% of the cell dry weight irrespective of the total lipid content (Rattray et al., 1975). The glycerophospholipid composition of numerous yeasts and yeast-like fungi has been reported (Kaneko et al., 1976; Ratledge and Evans, 1987; Weete, 1980). The five principal glycerophospholipids are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and diphosphatidylglycerol (DPG;

cardiolipin). There is a remarkable similarity in the relative proportions of these major glycerophospholipids among yeast species. With few exceptions PC is the major glycerophospholipid of yeasts, representing 25 to 55% of the total. Sometimes PE is predominant, but usually is the second most abundant glycerophospholipid, representing 13 to 38% of the total. PI ranges from 7 to 21% of total, PS ranges from 4 to 19%, and DPG ranges from 1 to 15%.

The most widely studied single yeast species with respect to glycerophospholipid composition is Sacch. cerevisiae. At least twelve glycerophospholipids have been isolated, and include PC, PE, PI and PS as the principal components. Minor components include phosphatidylmonomethylethanolamine (PMME), phosphatidyldimethylethanolamine (PDME), phosphatidic acid (PA), lysophosphatidylethanolamine (LPE), phosphatidylglycerol (PG) and DPG along with two unidentified phosphorus-containing lipids (Getz et al., 1970; Letters, 1966; Steiner and Lester, 1972). It is however, generally accepted that many of these minor components could arise by the uncontrolled action of phospholipases during lipid extraction (Ratledge and Evans, 1987).

Few molecular species of individual glycerophospholipids have been identified, but 1,2-dipalmitoleoyl-sn-glycerol-3-phosphorylcholine has been identified in Candida sp., Hanseniaspora valbyensis, Sacch. carlsbergensis and Sacch. cerevisiae (Hunter and Rose, 1971; Weete, 1980). As phospholipids are involved in membrane structures and functions there is a requirement for them to be fluid rather than crystalline. Consequently, the degree of unsaturation

in the overall fatty-acyl groups is generally higher than is found in triacylglycerols (Haley and Jack, 1974; Phornpiboonya and Jack, 1980; Suzuki and Hasegawa, 1974b, c; 1976).

Sterols and sterol esters. The determination of specific sterols is a problem and is hampered by the lack of authentic standard compounds. However ultraviolet absorption spectroscopy and GLC-mass spectrometry are reliable procedures for identification purposes (Weete and Laseter, 1974).

Most of the detailed work on yeast sterols has been carried out with Sacch. cerevisiae though there are the occasional analyses of sterols in other yeasts. The total sterol component of yeasts has been generally determined to range from 1 to 10% of total cell lipid, and ergosterol has been commonly identified as the major sterol in Sacch. cerevisiae (Henry, 1982), Kluyveromyces fragilis (Penman and Duffus, 1974) and C. albicans (Čapek et al., 1974), accounting for over 90% of the total.

The next most common sterol found in yeasts is the precursor of ergosterol, 24(28)-dehydroergosterol, first reported in baker's yeast by Breivik et al. (1954). This was found to be the major sterol in Sacch. cerevisiae NCYC 366 by Longley et al. (1968), though Hunter and Rose (1972) using the same strain found ergosterol and 24(28)-dehydroergosterol in about equal proportions. Zymosterol has also been reported in many yeasts, mainly in the esterified form (Hossack et al., 1977; Marriott, 1975). Other minor sterols reported in yeasts include lanosterol, 7,22-ergostadien-3-β-ol, episterol, fecosterol, mono- and di-methylzymosterol, and

4α-methyl-8,24(28)-ergostadien-3β-ol, some of which are intermediates in ergosterol biosynthesis (Aries and Kirsop, 1978; Henry, 1982; Hunter and Rose, 1971; 1972).

Sterols occur both in the free form and as esters with long-chain fatty acids. Both forms appear to be freely interconvertible though they have distinct functions; free sterols are associated with membrane functions, while sterol esters may be biosynthetic intermediates or fulfil a storage or 'pool' function (Bailey and Parks, 1975). Esterified derivatives of various sterol biosynthetic intermediates have been observed in Sacch. cerevisiae (Parks et al., 1974), and many are characterized by a high content of $C_{16:1}$, $C_{18:1}$ and, in certain genera, $C_{18:2}$ fatty-acyl residues (Hunter and Rose, 1972; Longley et al., 1968; Madyastha and Parks, 1969).

Carotenoids. Carotenoids are pigmented tetraterpenes (C_{40}) consisting of eight isoprene units. Hydrocarbon carotenoids are called carotenes and those containing oxygen are called xanthophylls. The principal yeasts whose carotenoids have been studied are, not surprisingly, the coloured ones, and like many other yeast lipids, the levels depend on the species and growth conditions (Weete, 1980). In the red yeasts, Rhodotorula and Rhodosporidium, the most abundant carotenoids are generally torulene and torularhodin. These carotenoids also exist in most Sporobolomyces/Sporidiobolus species, while other carotenoids such as β-, γ- and δ-carotene, phytoene, phytofluene, β-zeacarotene, plectaniaxanthin and astaxanthin have also been reported in several other yeasts (Goodwin, 1980; Simpson et al., 1971).

Sphingolipids and glycolipids. Sphingolipids occur as minor components of many yeasts; usual contents being less than 0.5% of the cell dry weight (Rattray et al., 1975; Weete, 1980). Sphingolipids are hydroxylated fatty-acid esters of long-chain amino-alcohols (sphingosines). Sphingosines occur as integral units of several yeast lipids ranging from relatively simple cerebrins, where the sphingosines are acylated with saturated, unsaturated or 2-hydroxy long-chain fatty acids (Nurminen and Suomalainen, 1971; Stanacev and Kates, 1963), to the complex phosphosphingolipids and glycophosphosphingolipids (Brennan and Lösel, 1978). Phospho-sphingolipids have been recovered from C. albicans and the yeast-like form of Pullularia pullulans. In addition, several glyco- and glycophospho-sphingolipids have been isolated from C. utilis and Sacch. cerevisiae. These include those containing phosphorylinositol, galactose, mannosylinositol and mannosyldi-inositoldiphosphate (Brennan and Lösel, 1978; Ratledge and Evans, 1987).

As well as the complex glyco- and glycophospho-sphingolipids, small quantities of other glycolipids have been found in yeasts. Simple acylated sugars such as acylglucose, or glycosylated sterols such as the steryl glycosides occur in Sacch. cerevisiae and other yeasts (Brennan et al., 1970; Parodi, 1977; Työrinoja et al., 1974). Acylated derivatives of the polyols, mannitol, arabitol and xylitol are produced as extracellular lipids of Rhodotorula species with 3-hydroxypalmitic and 3-hydroxystearic acids being the predominant acyl residues (Stodola et al., 1967; Tulloch and Spencer, 1964). Also occurring as extracellular glycolipids, and of

some commercial interest, are the sophorose-containing lipids produced by Torulopsis (Candida) bombicola and C. bogoriensis (Spencer et al., 1979). These glycolipids are based on sophorose to which is attached a hydroxy fatty acid via a glycosidic link. In C. bombicola, the hydroxy group is in either the ω or ω-1 position and the sophorolipid may also occur in the lactonized form (Göbbert et al., 1984; Ito and Inoue, 1982).

Other lipids. Hydrocarbons are probably the least understood of the various classes of yeast lipids. Quantitatively, these compounds account for 2 to 20% of the total lipid in some yeast strains (Barron and Hanahan, 1961; Kovác̆ et al., 1967). These can be both saturated and unsaturated, and range from C_{15} to C_{39} in chain length. A recent example is that of Guerzoni et al. (1985) who reported straight-chain alkanes in C. steatolytica ranging from C_{21} to C_{35} in length. Derivatives of alkane oxidation, such as mono- and di-hydric alcohols do occur in yeasts (Britton, 1984; Fukui and Tanaka, 1981; Ratledge, 1980; Rehm and Reiff, 1981). These long-chain alcohols have been shown to be mainly present as components of wax esters (Davidova et al., 1978; Muratov et al., 1979; Zalashko and Salokhina, 1982; Zalashko et al., 1979). This subject is covered in further detail in the section entitled 'Fatty-Acid Metabolism and Synthesis of Long-Chain Alcohols by Yeasts'.

Polyprenols, which are constituents of certain bacterial cell-wall polymers (Douglas and Baddiley, 1968; Scher et al., 1968), have been isolated from baker's yeast as a mixture of dolichols ranging in chain length from C_{70} to C_{105}. These probably occur in

most yeasts (Dunphy et al., 1967). Amongst other unusual, but naturally occurring, lipids of yeasts are various diol lipids. Bergel'son et al. (1966) originally isolated a number of such lipids from L. starkeyi. These were long-chain diesters of dihydric alcohols and 1-alkenyl ethers of their mono-esters. The latter class of compounds are analogues of neutral plasmalogens. Plasmalogens have been detected in P. pullulans, where they are present in both the neutral lipid and glycerophospholipid fractions (Goni et al., 1978).

Subcellular Distribution

Cellular organelles of eukaryotic organisms invariably differ in their lipid composition. Membranes are the main structural features of most subcellular organelles and, therefore, the relative proportions of the various lipids comprising these membranes reflect the overall cellular composition (Daum, 1985; Hunter and Rose, 1971; Weete, 1980).

Saccharomyces cerevisiae and other yeasts are typical eukaryotic organisms with respect to the membranous organelles they contain as well as the lipids that exist in these membranes. The mitochondrion, vacuole, endoplasmic reticulum, nucleus and plasma membrane are the membranous organelles of most yeasts and they contain a typical mixture of phospholipids, including PC, PE, PI, PS and DPG (Getz et al., 1970; Henry, 1982; Jakovcic et al., 1971; Trivedi et al., 1982), and sterols (Henry, 1982). Depending on the stage of growth other lipids may accumulate as globules, which are believed to be mainly composed of triacylglycerols. These globules

are often referred to as lipid granules, liposomes, spherosomes or low-density vesicles (Brennan and Lösel, 1978; Cartledge and Rose, 1973; Weete, 1980).

At present, not all of these different organelles and membranes have been successfully isolated from yeasts in an undamaged and purified form. Considerable progress, however, has been made in developing techniques for the isolation and purification of fractions enriched in plasma membrane.

Plasma membranes. Procedures used to isolate the plasma membrane have employed three main strategies.

1. Following the enzymic removal of the yeast cell wall, osmotically sensitive spheroplasts are lysed to release plasma-membrane vesicles which can be subsequently enriched by density-gradient centrifugation (Longley et al., 1968; Schibeci et al., 1973). A recent variation of this approach uses concanavalin A to stabilize the spheroplast, which results in more intact plasma membrane being recovered after lysis (Santos et al., 1978; 1982). An elegant and very useful modification of this method for the isolation of plasma-membrane preparations has come from Jacobson's laboratory in the United States (Chaney and Jacobson, 1983; Schmidt et al., 1983). This procedure has been used to isolate plasma membrane from Sacch. cerevisiae, and relies on the removal of the cell wall followed by the attachment of cationically-charged silica microbeads to the outer-surface of the spheroplasts (Cartwright, 1986; Cartwright et al., 1987; Schmidt et al., 1983). These beads enhance plasma-membrane density and stabilize it against

vesiculation, allowing large open sheets of plasma membrane to be isolated by low-speed centrifugation upon spheroplast lysis.

2. Another strategy involves whole cell homogenization followed by differential and density-gradient centrifugation (Fuhrmann et al., 1974; Wehrli, 1975) or zonal centrifugation (Nurminen et al., 1976; 1977). This may be followed by enzymic removal of wall material from the isolated cell-envelope fraction (Nurminen et al., 1970). It has, however, been reported that this method of cell rupture and subsequent isolation of plasma membrane yields unclean preparations, since other subcellular organelles appear to be entrapped (Dubé et al., 1973).

3. An entirely different approach developed by Fuhrmann et al. (1976) uses an adjustment of vesicle preparations to pH 4 to aggregate and remove non-plasma membrane components from the non-aggregated plasma-membrane vesicles.

Boulton (1965) analysed the gross composition of membranes obtained by controlled lysis of spheroplasts followed by differential centrifugation. Plasma membranes isolated by this method contained roughly equal proportions of lipid and protein (45 to 50%, by weight) with carbohydrate making up the remaining 5 to 10%. The results of subsequent investigations employing sucrose-density centrifugation as an additional membrane purification step were in agreement with Boulton's analysis (Longley et al., 1968; Schibeci et al., 1973). Longley et al. (1968), using Sacch. cerevisiae, first reported on the qualitative distribution of the major lipid classes in a poorly identified plasma-membrane preparation. They noted the presence of the neutral lipids mono-,

di- and tri-acylglycerols, sterols, sterol esters and free fatty acids. Phospholipids present included PC, LPC, PE, PI and PS. Plasma membranes were richer in PE, PI and PS compared with whole organisms. The principal fatty-acyl residues of all major lipid classes were $C_{16:0}$, $C_{16:1}$ and $C_{18:1}$. Suomalainen and Nurminen (1970), also working with Sacch. cerevisiae, confirmed the enrichment for PI and PS and the abundance of $C_{16:0}$, $C_{16:1}$ and $C_{18:1}$ residues in a plasma-membrane fraction characterized by a Mg^{2+}-ATPase marker enzyme. Cartwright (1986) and Cartwright et al., (1987) found that the relative proportions of fatty-acyl residues within the plasma-membrane phospholipids were similar to those reported by Beavan et al. (1982) for whole-cell phospholipids.

Although much work has been done on the lipid composition of the yeast plasma-membrane, there are considerable discrepancies in the literature concerning the relative contribution of various lipid classes to the overall lipid composition of plasma membranes obtained from Sacch. cerevisiae. Kramer et al. (1978) reported that the phospholipid content of yeast plasma-membranes was very low, comprising only 5 to 6% of the total plasma-membrane lipid. However, Kaneko et al. (1976) found that phospholipids constituted over 50% of the total cellular lipid of Sacch. cerevisiae and Nurminen et al. (1976), growing this organism under glucose-repressed conditions, reported that over 80% of the total cellular phospholipid and sterol was found in the plasma-membrane fraction. In view of the role of phospholipids within plasma-membrane bilayers (Singer and Nicolson, 1972), the proportions found by these latter two groups are much more realistic than the very low proportions reported by Kramer et al. (1978).

Low-density vesicles. While it is known that phospholipids and sterols are located in membranes, much less has been reported on the intracellular location and metabolic role of neutral lipids in yeasts. Light-microscope observations by Guilliermond et al. (1933) established that Sacch. cerevisiae contained one or more large organelles, termed vacuoles, as well as a larger number of smaller structures which, since they stained with lipid-soluble dyes, were referred to as lipid granules (Guilliermond, 1923). Bauer et al. (1975), working with the same species also deduced from freeze-etching techniques and frozen ultra-thin sectioning the presence of lipid granules.

Isolation of lipid granules, from yeast cell homogenates or lysates, purified using flotation techniques through density-gradients and subsequent analysis has confirmed their lipid nature (Cartledge et al., 1977; Clausen et al., 1974; Hossack et al., 1977; Schaffner and Matile, 1981). Clausen et al. (1974) isolated low-density vesicles from mechanically disrupted cells of Sacch. cerevisiae and found that triacylglycerols and esterified sterols were the major lipid components, accounting for 90 to 95% of the total vesicular lipid. The lipid-rich yeast L. starkeyi has also been reported to contain globules rich in triacylglycerols, which are probably similar if not identical to the vesicles found in Sacch. cerevisiae (Uzuka et al., 1975).

Many of these workers have suggested that low-density vesicles are physiologically connected with other membranes, possibly transferring material for their biosynthesis. A clue as to the role of intracellular vesicles in the physiology of Sacch. cerevisiae

came from a report by Sentandreu and Northcote (1969) and then Wiemken et al. (1970) showing that, during the cell cycle, large vesicles fragment to produce smaller vesicles which then fuse with the growing regions of the cell envelope. Cartledge et al. (1977) isolated two classes of low-density vesicles from Sacch. cerevisiae, small (0.43 μm diameter) and large (0.62 μm diameter). In addition to being rich in neutral lipids, the two classes of vesicles contained lipase, β-glucanase and α-mannosidase activities. The presence of these enzymes provides evidence for a vesicular role in envelope growth, since they could be involved in breakdown of wall components before insertion of new wall material (Cartledge et al., 1977; Cortat et al., 1972; Matile et al., 1971). Henschke et al. (1983) reported more evidence for the association of low-density vesicles with the plasma membrane. Using the same strain of Sacch. cerevisiae as that used by Cartledge et al. (1977), they isolated membranes with associated low-density vesicles from a randomly dividing population of organisms. Using ^{125}I-labelled spheroplasts these membranes were identified as being plasma membrane.

Mitochondria and other subcellular organelles. The sterol content of inner and outer mitochondrial membranes isolated from Sacch. cerevisiae has been examined by Bottema and Parks (1980). In contrast to reports that sterols in other organisms are located exclusively in the outer mitochondrial membrane (Comte et al., 1976; Hallermayer and Neupert, 1974), Bottema and Parks (1980) found no difference in the phospholipid/sterol ratio of the two

membranes. The phospholipid composition of the mitochondrial membranes derived from Sacch. cerevisiae has been found to be related to the degree of mitochondrial activity (Jakovcic et al., 1971) in that the proportion of DPG is greater in respiratory-sufficient, as compared with respiratory-deficient, yeast strains.

Although other subcellular organelles such as nuclei (Rozijn and Tonino, 1964), endoplasmic reticulum (Swida et al., 1982) and vacuoles (Emter and Wolf, 1984; Indge, 1968) have been isolated from yeast cells, their lipids have not been fully characterized.

Cell wall. The yeast cell wall, although not being a membranous structure, has been shown to contain a lipid component ranging from less than 2 to 13.5% (w/w; Phaff, 1971; Weete, 1980). Nurminen and Suomalainen (1969) and Suomalainen and Nurminen (1970) reported that the cell-wall lipids from Sacch. cerevisiae were composed of neutral lipids (including sterols) and glycerophospholipids with $C_{16:1}$ and $C_{18:1}$ as the principal fatty-acyl residues. Bianchi (1967) working with C. albicans found 40 to 60% of the cell-wall lipid to be sterol and sterol ester, regardless of its morphological form. However, because of the isolation procedures adopted to obtain cell-wall preparations (Domer and Hamilton, 1971; Safe and Caldwell, 1975) the possibility of contamination with lipid-rich plasma membrane should always be considered.

Effect of Carbon Source and Oxygen Tension on Lipid Content

Conditions for optimal growth vary with different yeasts and even different strains of the same species. The balance of

metabolic activities can be altered by manipulation of the culture conditions to favour production of a certain product, often at the expense of the production of other substances and even growth. The lipid composition of yeasts, and other micro-organisms for that matter, is very sensitive to changes in the chemical and physical properties of the environment. Among the environmental factors that have been reported to affect lipid composition of micro-organisms are growth rate, composition of the medium, growth temperature and dissolved oxygen tension (Babij et al., 1969; Brown and Rose, 1969; Chopra and Khuller, 1983; Prasad, 1985; Ratledge, 1982). In addition to various environmental factors, the nature of the carbon source has a pronounced effect on the quantity and composition of yeast lipid (Rattray et al., 1975), although the underlying mechanisms involved are far from understood.

All yeast species studied so far are able to use glucose as the sole source of carbon and energy (Barnett, 1976). Consequently, utilization of glucose by different yeasts has been studied most extensively (Fiechter et al., 1981; Käppeli, 1986; Käppeli and Fiechter, 1982; Käppeli et al., 1985a, b). There is, however, much controversy over the classification of yeasts according to their glucose metabolism. For a long time, two main categories of yeast have been differentiated on the basis of their sensitivity to free glucose. Yeasts in the first group are glucose-sensitive (Crabtree-positive). Their respiration is repressed in the presence of low concentrations of the free sugar, ethanol accumulates to large concentrations under strong glucose repression, formation of biomass is drastically decreased and many can grow anaerobically

under favourable nutritional conditions. This group is represented by species of Saccharomyces, Schizosaccharomyces, Debaryomyces and Torulopsis. The second group of yeasts are insensitive to free glucose (Crabtree-negative). These show relatively fast growth and high yields of biomass under unrestricted oxygen supply. Ethanol is not excreted and they cannot grow in the absence of oxygen. Typical representatives are found in the genera Candida, Rhodotorula, Trichosporon, Pichia, Torulopsis and Hansenula. Data from taxonomic studies indicating absence of ethanol formation may, however, be misleading because rigorous testing under strong glucose repression together with oxygen limitation has not always been carried out (Fiechter et al., 1981). Under such conditions numerous Candida species, for instance, show a repressed metabolism (Einsele et al., 1972; Johnson et al., 1972; Skipton et al., 1974). These may be referred to as intermediary yeast-types with respect to glucose sensitivity (Fiechter et al., 1981). With such high concentrations of glucose (greater than 75 g l^{-1}) it is difficult to determine the reason for ethanol release. External parameters (especially oxygen-transfer limitation) may influence the physiology of these yeasts leading to erroneous conclusions.

Käppeli (1986), in his studies on regulation of carbon metabolism in Sacch. cerevisiae and related yeasts, found that respiration is never completely absent during aerobic fermentation on glucose, pointing out that the so-called 'Crabtree effect' applied to yeast by De Deken (1966) does not adequately describe respiro-fermentative glucose metabolism. He suggests yeasts may be grouped according to their glucose metabolism under carbon and

oxygen limitations as follows: (1) purely respiratory yeasts (e.g. Trichosporon cutaneum; Käppeli and Fiechter, 1982) not showing ethanol formation even under oxygen limitation; (2) oxygen-sensitive yeasts (e.g. C. tropicalis; Fiechter et al., 1981) which produce ethanol under oxygen limitation; and (3) glucose-sensitive yeasts exhibiting aerobic ethanol production in the presence of excess glucose (e.g. Sacch. cerevisiae; Fiechter et al., 1981). Despite the observations made by Käppeli and his co-workers (Fiechter et al., 1981; Käppeli, 1986; Käppeli and Fiechter, 1982; Käppeli et al., 1985a, b), the expressions 'Crabtree-positive' and 'Crabtree-negative' still appear to be used most frequently, if only by convention, to describe a yeast's ability to metabolize glucose.

Brown and Johnson (1970) found that increasing the glucose concentration from 2 to 10 g l^{-1} in chemostat cultures of Sacch. cerevisiae had a marked effect on the organism's fatty-acyl composition. The total content of fatty-acyl residues fell from 8 to 5% dry weight and there was a disproportionate decrease in the content of unsaturated components. They also correlated such changes with a decrease in mitochondrial lipid, although this could have possibly been due to a simultaneous state of anaerobiosis brought on by high concentrations of glucose (Lowden et al., 1972). In contrast, Babij et al. (1969) reported that in C. utilis there was an accumulation of lipid as the concentration of glucose in the medium was increased. Results presented by Johnson et al. (1972) confirmed and extended those of Babij et al. (1969) and Brown and Johnson (1970). They found that fatty-acyl contents of Sacch.

cerevisiae, Sacch. carlsbergensis and Sacch. debruechii decreased with increasing concentrations of glucose, and referred to these as being Crabtree-positive yeasts. These lowered values were due to a lower content of sterol esters and phospholipids in Sacch. cerevisiae, and of sterol esters in the other yeasts. Fatty-acyl contents of Sacch. fragilis, Schwanniomyces occidentalis and C. utilis, referred to as Crabtree-negative yeasts, increased with increasing concentrations of glucose and in C. utilis this was due almost entirely to a higher content of triacylglycerols. It was suggested by these workers that the observed changes in lipid composition may represent differences in the metabolism of organisms which differ with respect to the Crabtree effect. In the case of Crabtree-positive species of Saccharomyces, an increase in glucose concentration resulted in repression of oxidative enzymes. This was shown by a lowered respiratory quotient (Q_{O_2}) and an increase in ethanol production. In the Crabtree-negative yeasts, there was no repression of oxidative enzymes and only small amounts of ethanol were detected when glucose concentrations were high.

Many other yeasts, also referred to as Crabtree-negative yeasts by Ratledge and Evans (1987), such as Candida 107 (Hall and Ratledge, 1977), L. starkeyi (Boulton and Ratledge, 1981), Rh. gracilis (Rolph et al., 1986) and Rh. glutinis (Yoon and Rhee, 1983), all have raised cellular lipid contents when glucose concentrations are increased, and these may be increased even further by limiting particular nutrients in the presence of excess carbon. In batch culture, growth ceases when the limiting nutrient, usually the principal nitrogen source, becomes exhausted whilst

excess carbon continues to be assimilated by the organism and channelled into lipid (Kessell, 1968; Ratledge, 1982).

Various other carbon sources such as xylose, maltose, lactose, fructose, mannitol, glycerol, ethanol, galactose and soluble starch have been used in lipid studies on yeasts (Brown and Johnson, 1970; Dostálek, 1986; Hansson and Dostálek, 1986; Tahoun et al., 1986). Lipid contents of Cryptococcus albidus from between 33% (w/w) with xylose to 44% (w/w) with glycerol and mannitol substrates have been reported by Hansson and Dostálek (1986). Tahoun et al. (1986) studied the effects of selected sugars on the lipid composition of Candida lipolytica and noted an increase in lipid content in galactose-, sucrose- and lactose-grown cells, reaching 7 to 12% (w/w) compared with only 4.4% (w/w) in glucose-grown organisms.

Considerable variations in total lipid content as well as fatty-acyl composition occur when yeasts are grown on hydrocarbons or fatty acids (Bell, 1973; Ratledge, 1980). With these substrates, the subsequent fatty-acyl groups of the yeasts' lipid reflect the chain length and, to a certain extent, the degree of unsaturation of the original carbon source (Britton, 1984; Markovetz and Kallio, 1964). Although the capacity to utilize hydrocarbons is generally non-existent in Saccharomyces species and many other yeasts (Markovetz and Kallio, 1964), it is an efficient process in certain genera, especially Candida and Torulopsis (Boulton and Ratledge, 1984; Klug and Markovetz, 1971). In particular, utilization of hydrocarbons by yeasts is heavily dependent upon an active respiratory system and the availability of oxygen (Boulton and Ratledge, 1984; Einsele et al., 1972). What is also often observed

with many alkane-grown yeasts as compared to glucose-grown organisms is a slow growth rate, a lowered yield of biomass, and a corresponding increase in total lipid content (Hug et al., 1974; Nyns et al., 1968; Ratledge, 1968). It is generally considered that a slow growth rate favours lipid accumulation since the rate of lipid synthesis is not controlled by growth of the organism (Ratledge, 1980; 1982). In contrast, various species of Candida grown on alkanes as opposed to glucose have been reported to contain a lower lipid content (Mishina et al., 1973; Thorpe and Ratledge, 1972). These differences may reflect varying metabolic capacities of different species, variations in environmental conditions or a combination of these effects.

Oxygen, like glucose, has a pronounced effect on the growth, general metabolism and lipid composition of yeast. Rates of synthesis of cellular components in species of Candida are controlled by the respiratory pathways (Einsele et al., 1972; Rogers and Stewart, 1974). Babij et al. (1969) and Brown and Rose (1969) discovered that oxygen availability governs the ability of C. utilis to synthesize polyunsaturated fatty acids. At high oxygen tension (160 μM), the occurrence of linolenoyl residues was greatest, reaching the same level as oleoyl residues (Babij et al., 1969), whereas at low oxygen tension the degree of unsaturation decreased and greater concentrations of C_{16} residues accumulated at the expense of C_{18} residues (Brown and Rose, 1969). The best controlled environment as far as oxygen tension is concerned has been achieved in chemostat cultures where the effect of oxygen on lipid production can be independently ascertained (Prasad, 1985;

Ratledge and Evans, 1987). It has been shown by Hall and Ratledge (1977) that the lipid content of chemostat-grown Candida 107 increased from 10.4 to 23.9% when the aeration rate was raised from 0.05 to 0.5 $vv^{-1}min^{-1}$. A similar pattern has also been observed for Rh. glutinis (Choi et al., 1982).

The lipid composition of aerobically grown as compared with anaerobically grown Sacch. cerevisiae has been reported by several workers (Ahvenainen, 1982; Bulder and Reinink, 1974; Hossack et al., 1977; Jollow et al., 1968; Kovác̆ et al., 1967; Watson and Rose, 1980). Anaerobically grown Sacch.cerevisiae has a lower total lipid content, a highly variable acylglycerol fraction and decreased contents of phospholipid and sterol (Jollow et al., 1968; Kovác̆ et al., 1967). The major differences in the phospholipid composition of anaerobically grown, as compared with aerobically grown organisms, are lowered proportions of DPG and PE and increased proportions of PC and PI, particularly when cultures are entering the stationary phase of growth (Getz et al., 1970; Jollow et al., 1968). Jollow et al. (1968) reported that 80 to 90% of the fatty-acyl residues associated with acylglycerols and phospholipids in aerobically grown Sacch. cerevisiae were palmitoleoyl and oleoyl residues. Anaerobically grown organisms were characterized by a high proportion (up to 50% of the total fatty-acyl residues) of shorter chain, saturated fatty-acyl residues ranging from C_8 to C_{14} and a low proportion of unsaturated fatty-acyl residues in the phospholipid fraction. The more recent findings of Ahvenainen (1982), on the lipid composition of aerobically and anaerobically propagated brewer's bottom yeast, are in accordance with those

found by earlier workers. Unsaturated residues constituted 86% of the total fatty-acyl residues in aerobically grown organisms but only 38% in those grown anaerobically. The principal residues in aerobically cultured cells were palmitoleoyl and oleoyl. Palmitoyl residues predominated in anaerobically cultured cells. The distribution of unsaturated residues were similar to those found by Jollow et al. (1968).

It has been long recognized that lipids, particularly unsaturated fatty acids, play an important role in determining the function and properties of mitochondrial enzymes in Sacch. cerevisiae (Haslam and Mahdawi, 1980; Walenga and Lands, 1975a, b). Several mitochondrial membrane-bound enzymes have been shown to require, or are associated with, phospholipids (Watson et al., 1975).

When strains of Sacch. cerevisiae are grown under strict anaerobic conditions, they become auxotrophic for a sterol (Andreasen and Stier, 1953) and an unsaturated fatty acid (Andreasen and Stier, 1954). These compounds cannot be synthesized in the absence of oxygen since the fatty-acyl desaturase enzyme and two of the enzymes catalysing the conversion of squalene to ergosterol require molecular oxygen. Other quantitatively minor anaerobically-induced requirements such as nicotinic acid (Suomalainen et al., 1965) are usually supplied by a low concentration of yeast extract (Alterthum and Rose, 1973). At one time it was thought that the requirements for both a sterol (Hossack and Rose, 1976; Proudlock et al., 1968) and an unsaturated fatty acid (Light et al., 1962) were fairly non-specific. While

this still may be true for unsaturated acids (Holub and Lands, 1975; Nes et al., 1984), it is not necessarily true for sterols (Nes et al., 1976; 1978; Pinto and Nes, 1983). These workers have demonstrated that the ability of different sterols to support anaerobic growth is not simply an all or nothing phenomenon as had previously been implied. The natural yeast sterol, ergosterol, was the most capable of supporting anaerobic growth. However, by comparing pairs of sterols differing in only one component, they were able to show that certain groupings in ergosterol appeared to have some functional significance in the yeast.

Lipid supplements are not, however, modified by the organism (Nes et al., 1978). This anaerobically induced auxotrophy has been exploited by many workers for changing the lipid composition, particularly fatty-acyl unsaturation, of the plasma membrane in relation to composition-function relationship studies (Calderbank et al., 1984; 1985; Keenan et al., 1982; ; Thomas and Rose, 1979; Thomas et al., 1978). Many of these workers have, however, only been interested in the extent to which phospholipids are enriched with the exogenously supplied unsaturated fatty acid. The supplemented fatty acid has been shown to account for between 50 and 69% of the residues within the phospholipids, depending on the unsaturated fatty acid supplement and the strain of Sacch. cerevisiae. Very little has been reported on incorporation of exogenous fatty acids into different types of lipid during anaerobic growth, or even on the subcellular distribution of incorporated fatty acids. Watson and Rose (1980) reported a high proportion of supplemented unsaturated fatty acid in the triacyl-

glycerol fraction from anaerobically grown Sacch. cerevisiae NCYC 366. Values for unsaturation (Δmol^{-1}; Kates and Hagen, 1964) were as high as 0.93 when oleic acid was the supplement. This was higher than the value for PE and PI and PS, but similar to that for PC. When polyunsaturated fatty acid supplements were used (linoleic or α-linolenic) the Δmol^{-1} values for triacylglycerols were considerably higher than those for any of the individual phospholipid classes, and it was suggested that this could well be a safety mechanism to stabilize fluidity in cellular membranes. These results, however, conflict quite dramatically with those found earlier by Hossack et al. (1977). Using the same strain of Sacch. cerevisiae grown under anaerobic conditions in the presence of oleic acid, Hossack and his co-workers reported a Δmol^{-1} value of 0.06 for the triacylglycerol fraction and only 0.73 for total phospholipids. Both groups of workers appeared to have used very different procedures for extraction of lipids from organisms. This could possibly explain the large discrepancy between the two sets of data.

What has become quite apparent is that strict precautions in the analytical procedures must be adopted to obtain reproducible results. In addition, methods must be standardized if any conclusions are to be drawn from comparing different studies on lipid composition.

FATTY-ACID METABOLISM AND SYNTHESIS OF LONG-CHAIN ALCOHOLS BY YEASTS

Fatty Acid Activation and Degradation

Once free fatty acids are present within the organism, whether they are released from lipids (triacylglycerols, phospholipids), produced by alkane- or fatty acid-utilizing organisms or incorporated from exogenously supplied fatty acids, they must be converted to their CoA esters. This process not only activates them for subsequent utilization but also diminishes their inherent toxic effects on many enzymes (Boulton and Ratledge, 1984; Fukui and Tanaka, 1981). Two distinct long-chain fatty acyl-CoA synthetases have been isolated from C. lipolytica following initial reports of the occurrence of this enzyme in alkane-grown yeasts by Duvnjak et al. (1970) and Trust and Millis (1970). These enzymes have been extensively studied (Hosaka et al., 1979; 1981; Kamiryo et al., 1977; Mishina et al., 1978a, b; Numa, 1981). One of them, termed acyl-CoA synthetase I, is ubiquitous in that it occurs both in glucose- and alkane-grown cells and is found within membranes of the cytoplasm, including the mitochondrial membranes. It is considered that this enzyme is involved in the direct transfer of fatty-acyl residues from CoA esters into cellular lipids. Acyl-CoA synthetase II, on the other hand, occurs only in alkane-grown cells and is found within microbodies called peroxisomes. It is believed that this enzyme serves to link fatty acids, arising from alkane oxidation or exogenously supplied fatty acids, to the fatty acid β-oxidation cycle which is itself located within peroxisomes (Numa, 1981). The two enzymes are immunologically distinct proteins

(Hosaka et al., 1979) and they both have different substrate specificities (Mishina et al., 1978b).

Quantitatively, the most important and universally distributed pathway of fatty acid degradation is β-oxidation. This process occurs in mitochondria and, under certain conditions such as growth on alkanes or fatty acids, may preferentially take place in peroxisomes (Dommes et al., 1983). β-Oxidation involves the successive removal of C_2-units from fatty acids after they have been activated to their CoA esters, resulting in a molecule of acetyl-CoA and an activated fatty acid two carbon atoms shorter than the original substrate.

While in many organisms β-oxidation may be a source of ATP through degradation of fatty acids, in those that grow on alkanes or fatty acids peroxisomal β-oxidation may simply be a way of disposing of an 'energy-rich, carbon-poor' substrate (Boulton and Ratledge, 1984; Ratledge and Evans, 1987). In C. tropicalis grown on alkanes, reoxidation of $FADH_2$ has been linked to the activity of catalase (Teranishi et al., 1974; Yamada et al., 1982) rather than the respiratory chain, and this enzyme has been purified from both peroxisomes and the cytosol (Yamada et al., 1982). This serves to produce heat rather than ATP which would be surplus to the organism's requirements (Boulton and Ratledge, 1984).

Other oxidative pathways involved in degradation of fatty acids exist, if only by inference. These include α- and ω-oxidation. Degradation of fatty acids by α-oxidation involves an NAD^+-linked, oxygen-dependent oxidative decarboxylation with liberation of carbon dioxide and an acid with one less carbon atom than the

starting material. Unlike β-oxidation, α-oxidation does not require activation of the fatty acid substrate with CoA nor does it appear to be linked with energy production (Gurr and James, 1980). It has not been demonstrated with any great certainty in yeasts though the system has been demonstrated in several plants (Galliard and Matthew, 1976; Gurr and James, 1980; Markovetz and Stumpf, 1972; Shine and Stumpf, 1974). It has, however been suggested to occur in yeasts by the appearance of fatty acids with an even number of carbon atoms after growth on odd chain-length alkanes (Ratledge, 1978). To account for these findings, either an unusual elongation system must occur, using propionate, or α-oxidation takes place followed by conventional C_2-elongation. Gill and Ratledge (1973) and Mishina *et al.* (1976a, b) have provided evidence against *de novo* fatty-acid synthesis continuing to operate in alkane-grown organisms.

Hydroxylation of fatty acids at the α-carbon may be considered as an intermediate step in α-oxidation (Fulco, 1974; Gurr and James, 1980). Chain elongation and 2-hydroxylation pathways, which are specific for very long-chain fatty acids (greater than C_{18}), have been found in yeasts (Fulco, 1967; Vesonder *et al.*, 1970). In addition to the chain elongation and 2-hydroxylation systems, Fulco (1967) found a relatively non-specific system for oxidative decarboxylation of 2-hydroxy acids in *C. utilis*. All 2-hydroxy fatty acids tested (C_{18} to C_{26}, containing an even number of carbon atoms) were converted, in part, to the unsubstituted acid containing one carbon atom less than the substrate. Decarboxylation was most efficient with 2-OH-C_{18} and least with 2-OH-C_{26} acids.

Another product arising from the decarboxylation reaction was an aldehyde containing one carbon less than the 2-hydroxy acid substrate. The aldehydes were detected in trace amounts with the shorter chain substrates but were the major products from decarboxylation of 2-OH-C_{24} and 2-OH-C_{26} acids. As in the case in many plants (Gurr and James, 1980), the aldehyde is presumably an intermediate between the 2-hydroxy acid substrate and the unsubstituted fatty acid which is the final product in the oxidative decarboxylation process.

ω-Oxidation in micro-organisms has been known to occur for some time (Klug and Markovetz, 1971; Shennan and Levi, 1974). Fatty acids arising from an alkane or an exogenous fatty acid substrate are ω-oxidized by certain yeasts which produce either ω-hydroxy fatty acids as constituents of extracellular lipids (Gorin et al., 1961; Ito and Inoue, 1982; Spencer et al., 1979; Stodola et al., 1967) or α,ω-dicarboxylic acids (Hill et al., 1986; Shiio and Uchio, 1971). The initial ω-hydroxylation is possibly catalysed by the same hydroxylase that is responsible for the primary attack on alkanes (Marchal et al., 1982).

ω-Hydroxylations in yeasts are characteristically of a mixed-function oxidase type, involving both molecular oxygen and NAD(P)H. One atom of the oxygen molecule is transferred to the substrate (producing the ω-hydroxy acid) while the other is reduced by NAD(P)H to water (Cardini and Jurtshuk, 1970; Ratledge, 1980). Marchal et al. (1982) using the yeast Saccharomycopsis lipolytica found that growth on n-alkanes induced a hydroxylation system which, in the presence of NADPH and oxygen, converted lauric acid

($C_{12:0}$) into ω-hydroxy lauric acid. Further oxidation of ω-hydroxy acids, involving NAD^+-linked alcohol and aldehyde dehydrogenases, respectively, results in formation of α, ω-dicarboxylic acids (Boulton and Ratledge, 1984; Ratledge, 1980; Ratledge and Evans, 1987).

ω-Oxidation contrasts with α- and β-oxidation in that the oxidative attack is remote from the carboxyl group, and that there is not a corresponding decrease in the chain-length of the fatty acid substrate. Like α-oxidation, however, the substrate does not require activation with CoA nor does it appear to be linked with energy production. Whereas β-oxidation is primarily a mitochondrial and/or peroxisomal energy-linked process which is capable of complete degradation of a fatty acid molecule, ω-oxidation enzymes are located in the endoplasmic reticulum (Gurr and James, 1980; Marchal et al., 1982) and the process inevitably stops at formation of a dicarboxylic acid.

Further degradation of long-chain α, ω-dicarboxylic acids by β-oxidation has been implied by several workers (Boulton and Ratledge, 1984; Hill et al., 1986). Whether this would involve formation of a CoA ester at one of the terminal carboxyl groups is open to question.

Occurrence and Mechanisms of Long-Chain Primary Alcohol Production

Long-chain alcohols occur as components of animal, higher plant and microbial lipids as free alcohols, precursors of glycerol ether lipids and in combination with fatty acids as wax esters (Harwood and Russell, 1984; Mahadevan, 1978). Among micro-organisms that synthesize long-chain alcohols, bacteria have been studied most

extensively (Allen et al., 1971; Baptist et al., 1963; Day et al., 1970; Naccarato et al., 1972; 1974; Raymond and Davis, 1960; Stewart and Kallio, 1959), and more recently many have been analysed for their wax ester content (DeWitt et al., 1982; Fixter and Fewson, 1974; Fixter and McCormack, 1976; Fixter et al., 1986; Geigert et al., 1984; Lloyd and Russell, 1983a, b; 1984; Russell, 1978; Russell and Volkman, 1980). Other alcohol-containing bacteria have been investigated for their industrial importance in, for example, degradation of oil spillage (Higgins and Gilbert, 1978). In oil-degrading micro-organisms, long-chain alcohols are produced as intermediates in terminal oxidation of alkanes, and may also be found esterified with long-chain fatty acids which are produced by further oxidation of the alcohols (Britton, 1984; Geigert et al., 1984; Ratledge, 1978; Singer et al., 1985). When grown on carbon sources other than alkanes, some bacteria synthesize long-chain primary alcohols by reduction of CoA esters of long-chain fatty acids which arise either from activity of the fatty-acid synthetase (Day et al., 1970; Lloyd and Russell, 1983a; Naccarato et al., 1972) or are supplied exogenously (Lloyd and Russell, 1983a; Naccarato et al., 1974).

Some yeasts are able to grow on alkanes (Boulton and Ratledge, 1984; Britton, 1984; Ratledge, 1978; 1980) and certain of these organisms may accumulate wax esters containing long-chain alcohols. These yeasts include C. guilliermondii (Muratov et al., 1979), C. tropicalis (Davidova et al., 1978) and Rh. glutinis (Zalashko and Salokhina, 1982; Zalashko et al., 1979). Reports of long-chain alcohol production by yeasts grown on non-alkane substrates are

rare. Davidova et al. (1978) reported that wax esters were detectable in glucose-grown C. tropicalis harvested from exponential-phase cultures, although their synthesis was more prolific when this yeast was grown on alkanes.

It is generally accepted that in the majority of hydrocarbon utilizing micro-organisms the initial metabolism of n-alkanes takes the form of mono-terminal oxidation as indicated:

$$CH_3(CH_2)_nCH_3 \rightarrow CH_3(CH_2)_nCH_2OH \rightarrow CH_3(CH_2)_nCHO \rightarrow CH_3(CH_2)_nCOOH$$

Oxidation to the primary alcohol is catalysed by a mixed-function oxidase system (mono-oxygenase or hydroxylase) which may be linked to one of several different electron-carrier systems. To date, two microbial hydroxylation systems have been well characterized. Probably the best known mono-oxygenase system is that of Pseudomonas oleovorans consisting of rubredoxin, an NADH-rubredoxin reductase and a ω-hydroxylase (Peterson et al., 1967). On the other hand, Corynebacterium sp. contains another system composed of cytochrome P-450 and an NADH-cytochrome c reductase (Cardini and Jurtshuk, 1968; 1970). It is this latter system that probably exists in yeasts (Gallo et al., 1971; 1973; 1976; Honeck et al., 1982; Lebeault et al., 1971; Liu and Johnson, 1971).

In C. tropicalis, an induced alkane hydroxylase, requiring n-alkanes with more than 14 carbon atoms, is specifically located within microsomes and contains an NADPH-cytochrome c reductase instead of the NADH-linked enzyme (Gallo et al., 1971; 1973; 1976). However this coenzyme requirement would appear to be genus- or even

species-dependent, since it has been found that NADH and not NADPH is active with the alkane oxidase from C. lipolytica, while in extracts prepared from C. rugosa either NADH or NADPH could be used (Boulton and Ratledge, 1984; Higgins and Gilbert, 1978; Ratledge, 1978). It should be recognized though that, in crude microsomal preparations, ATP-dependent transhydrogenases may be present that lead to substitution of NADH for NADPH (Gallo et al., 1973).

Intermediates produced during alkane oxidation do not accumulate to any great extent and consequently quantitative values are not often quoted. However, there have been several attempts to increase the quantities of primary alcohols produced by alkane-utilizing micro-organisms, by careful control of oxidizing conditions (Fredricks, 1967) or by producing mutants blocked at an appropriate point in the oxidative pathway (Jenkins et al., 1972; Macham and Hedyeman, 1974). Although such mutants have been produced in bacteria and yeasts (Ratledge, 1980), it appears that a successful commercial process for production and accumulation of long-chain primary alcohols has still yet to be developed.

Another promising approach might be one based on exploitation of the ability of certain yeasts to metabolize long-chain fatty acids to yield long-chain primary alcohols. Although such a system has not yet been reported, the findings of Davidova et al. (1978) on production of wax esters in glucose-grown C. tropicalis may suggest that one does exist. Long-chain aldehydes have been reported in C. utilis as a result of α-hydroxylation and subsequent decarboxylation of fatty acid substrates (Fulco, 1967), but further reduction of these compounds yielding alcohols with one carbon atom

less than the original substrate was not reported.

Enzymic reduction of fatty acids has been shown to be the major pathway for production of primary alcohols in many other organisms (Mahadevan, 1978; Riendeau and Meighen, 1985), and it is possible that a similar processing route may exist, or be developed, in some yeast strains. Reduction of fatty acids appears to proceed via their activation with CoA followed by reduction with NADH or NADPH. A system involving acyl-CoA and fatty aldehyde as intermediates could be catalysed by the sequential reactions of acyl-CoA synthetase, acyl-CoA reductase and aldehyde reductase (see Fig. 1). Acyl-CoA reductases and aldehyde reductases have been found associated with microsomal membranes of Euglena gracilis (Khan and Kolattukudy, 1973; 1975; Kolattukudy, 1970), Mycobacterium tuberculosis (Wang et al., 1972), gourami roe (Griffith et al., 1981), and many mammals (Bishop and Hajra, 1978; 1981; Bourre and Daudu, 1978; Wykle et al., 1979). In other organisms, such as Clostridium butyricum (Day and Goldfine, 1978; Day et al., 1970), Escherichia coli (Naccarato et al., 1974) and Brassica oleracea (Kolattukudy, 1971), these enzymes have been located in the soluble protein fraction. In general, microsomal acyl-CoA reductases show a remarkably high specificity for NADPH, the only clear exception being the NADH-dependent system from E. gracilis (Khan and Kolattukudy, 1973; 1975; Kolattukudy, 1970). In contrast, the soluble systems are generally NADH specific with a few notable exceptions (Riendeau and Meighen, 1985). The aldehyde reductases may be NADH- or NADPH-dependent and may or may not differ in specificity from the acyl-CoA reductase (Day and Goldfine, 1978;

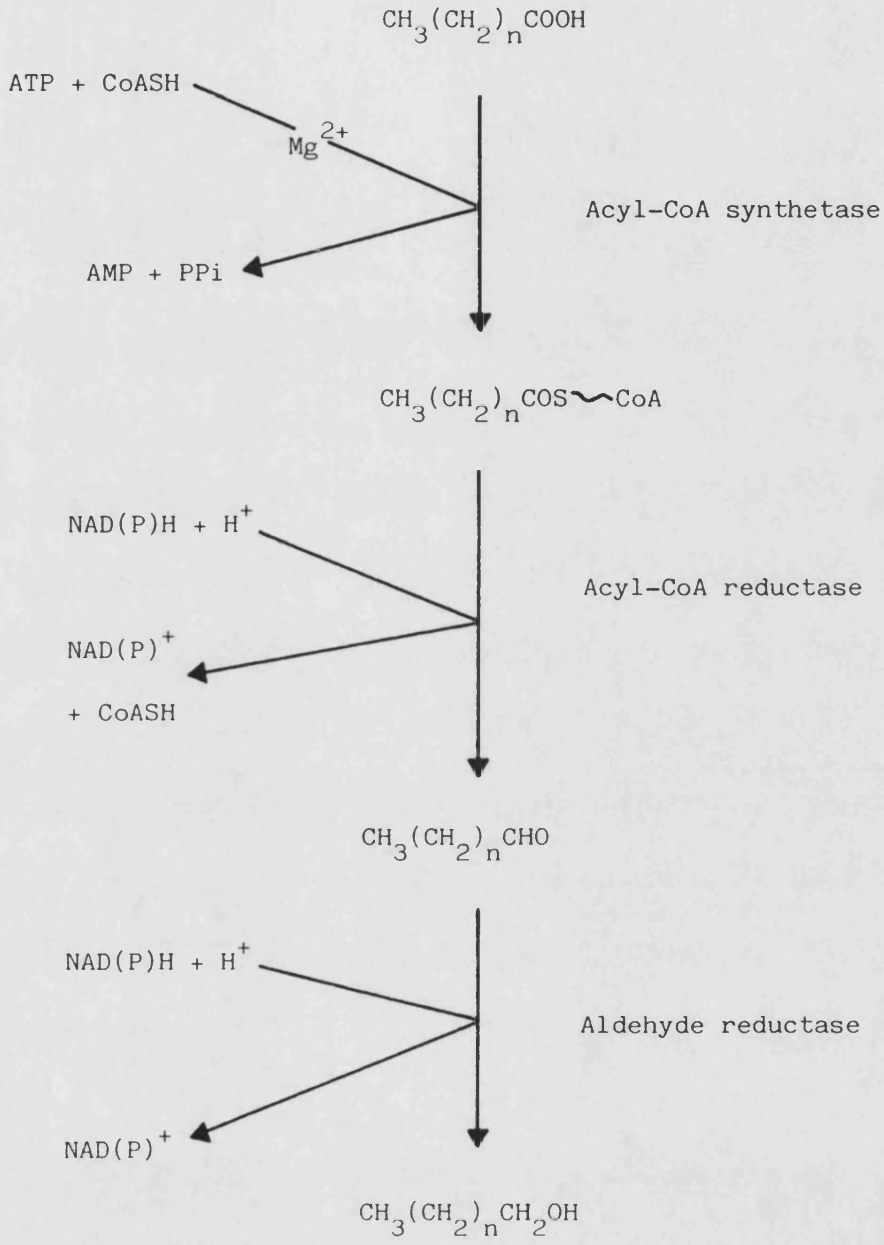


Figure 1. Reduction of fatty acids to primary alcohols

Kolattukudy, 1971; Riendeau and Meighen, 1981).

In addition to showing dinucleotide specificity, many of the isolated enzyme systems show varying degrees of fatty-acid and fatty acyl-CoA specificity. Maximal rates of fatty-acid reduction to primary alcohol in microsomes from E. gracilis are obtained for $C_{14:0}$ and $C_{16:0}$ acids with lower rates of conversion being observed for fatty acids of shorter or longer chain-lengths. The specificities for saturated and mono-unsaturated acids are also consistent with the chain lengths of the alcohol moieties found in the wax esters (Kolattukudy, 1970). The acyl-CoA reductase from Clostridium butyricum is relatively non-specific for chain length of its substrate with less than a two-fold variation in specific activity for $C_{12:0}$, $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ acyl-CoAs (Day and Goldfine, 1978).

COMMERCIAL IMPORTANCE OF LONG-CHAIN ALCOHOLS

Long-chain primary alcohols are valuable raw materials for the manufacture of surfactants, lubricants, cosmetics, and various other products. They are currently produced industrially either synthetically from petroleum products (olefins) or from natural oils and fats by various processes (Buchold, 1983; Mahadevan, 1978).

When low-cost crude oil was available, petrochemical processes were preferred over those based on natural oils and fats. The Oxo process or hydroformylation involving the reaction of an olefin with carbon monoxide and hydrogen gas in the presence of a cobalt catalyst at temperatures of 75 to 200°C and a pressure of 100 to

300 atm. ($1.01 \times 10^7 - 3.04 \times 10^7$ Pa) is an industrial method for preparing alcohols from olefins. This process yields alcohols with chain lengths ranging from C_{12} to C_{15} (Mahadevan, 1978). Another widely used petrochemical process is the Ziegler process, producing a wider range (C_2 to C_{20}) of alcohols (Mahadevan, 1978).

Due to the rapid escalation in the cost of mineral oils in comparison with that of vegetable and animal oils and fats, processes for production of long-chain alcohols from fatty acids have become increasingly important to the detergents and chemicals industries. When fats and oils from tallow, coconut oil and palm oil are the raw materials, long-chain alcohols are obtained by high-pressure hydrogenation of fatty acids, their esters or metallic salts. In general, this reduction is achieved with hydrogen gas at temperatures from 50 to 350 °C and pressures from 10 to 200 atm. ($1.01 \times 10^6 - 2.03 \times 10^7$ Pa) or more, in the presence of a catalyst (Buchold, 1983; Mahadevan, 1978). The range of alcohols produced by this process depends largely on the fatty-acid composition of the raw substrate used (Buchold, 1983).

All of these industrial processes have restrictions in terms of chain-length distribution and the inability to provide unsaturated long-chain alcohols, while the energy-intensive conversion makes the routes from oils, fats and petrochemicals relatively expensive. There is therefore a potential interest in alternative routes if these would provide flexibility in tailor-making various alcohol types at a lower or comparable cost to that of current pathways.

The first aim of the research reported in this thesis was to extend our understanding of fatty-acid incorporation into Sacch. cerevisiae grown under anaerobic conditions. The second, and major part of this study was to examine the ability of certain yeasts to produce long-chain alcohols and their potential to convert exogenously supplied fatty acids into these alcohols.

METHODS

ORGANISMS

The yeasts used were Candida albicans NCYC 1467, C. bombicola NCYC 1449, C. maltosa Colworth Microbial Culture Collection (CMCC) 3152, C. ingens NCYC 822, C. utilis NCYC 168, C. utilis NCYC 707, Debaryomyces hansenii NCYC 9, Pichia fermentans NCYC 850, Rhodotorula glutinis NCYC 59, Rh. glutinis CMCC 2272, Rh. rubra NCYC 195, Rhodotorula sp. ATCC 20254, Saccharomyces cerevisiae Y185 (a gift from J.R. Woodward) and Torulopsis petrophilum ATCC 20225. They were maintained at 4°C on slopes containing (l^{-1}): malt extract 3.0 g, yeast extract (lab m) 3.0 g, glucose 10 g, peptone 0.5 g and agar 20 g (MYGP; Wickerham, 1951).

EXPERIMENTAL CULTURES

Growth of Organisms for Studies on Long-Chain Alcohol Production

Organisms were grown aerobically (Patching and Rose, 1969) in a medium containing (l^{-1}): glucose 20 g, $(NH_4)_2SO_4$ 3.0 g, KH_2PO_4 4.5 g, yeast extract (lab m) 1.0 g, $MgSO_4.7H_2O$ 25 mg and $CaCl_2.2H_2O$ 25 mg, adjusted to pH 4.5 with HCl. Growth under self-induced anaerobic conditions (Beavan et al., 1982) used the same medium except that the concentration of glucose was 200 gl^{-1} and that of yeast extract 4.0 gl^{-1}. Where indicated (i) the concentration of glucose was altered to 10 - 300 gl^{-1} or replaced with either 100 g galactose or glycerol l^{-1} and that of yeast extract altered to 4.0 gl^{-1}, (ii) cultures were supplemented with various long-chain fatty acids (10 mgl^{-1}) either at zero time or fed 2 mg $(24\ h)^{-1}$

during the stationary phase of growth to a final concentration of 10 mgl^{-1}. Supplements were added as solutions in AnalaR ethanol. One-litre portions of medium were dispensed into 2 l round flat-bottomed flasks and sterilized by autoclaving at 10 lb in^{-2} (6.89×10^4 Pa) for 1 min. Flasks used for aerobic cultures were plugged with cotton wool, and those used for self-induced anaerobic cultures were fitted with fermentation locks (Beavan et al., 1982). Starter cultures (100 ml of the appropriate medium in a 250 ml flask) were inoculated with a pinhead of organisms from a slant culture, and incubated for 48 h at 30°C on an orbital shaker (200 r.p.m.). Experimental cultures used in the survey of long-chain alcohol production were inoculated with 1 ml of starter culture; those used for further studies were inoculated with a portion of starter culture containing 10 mg dry wt organisms. Cultures were incubated in a constant temperature room (30°C) with stirring (300 r.p.m.) on a magnetic flat-bed stirrer.

Growth was followed by measuring optical density at 600 nm, measurements being related to dry wt of each yeast strain using an appropriate calibration curve. At times indicated, organisms were harvested by centrifugation (6000 g; 1 min; 4°C) and washed twice with water. All centrifugation regimes were carried out in a Sorvall RC5C refrigerated Superspeed Centrifuge (Du Pont Company, Wilmington, Delaware, U.S.A.) unless otherwise stated.

Growth of Organisms for Studies on Oleic Acid Incorporation

Saccharomyces cerevisiae Y185 was grown anaerobically by a modification of the method of Alterthum and Rose (1973) in medium

containing (l^{-1}): glucose 50 g, $(NH_4)_2SO_4$ 3.0 g, KH_2PO_4 4.5 g, yeast extract (lab m) 1.0 g, $MgSO_4.7H_2O$ 25 mg and $CaCl_2.2H_2O$ 25 mg, adjusted to pH 4.5 with HCl. One-litre portions of medium were dispensed into 2 l round flat-bottomed flasks and sterilized as previously described. Anaerobic conditions were maintained throughout growth by flushing the flasks with high-purity nitrogen gas from which the last traces of oxygen had been removed by a column-type oxy-trap together with a self-indicating oxy-trap (Alltech Associates Incorporated, Deerfield, Illinois, U.S.A.). Prior to inoculation, the medium was supplemented with ergosterol (5 mg l^{-1}) and oleic acid (30 mg l^{-1}). Supplements were made up in AnalaR ethanol. Where indicated, cultures were supplemented with an additional 2.5 µCi [1-^{14}C]oleic acid [201 µCi (7.43 MBq) mg^{-1}] either at the start of growth or at various times before harvesting. Portions of medium were inoculated with 1 mg dry wt organisms from an overnight starter culture and incubated as previously described. Growth was followed as already described, and organisms were harvested from mid exponential-phase cultures (0.24 - 0.26 mg dry wt ml^{-1}) by centrifugation (6000 g; 1 min; 4°C). Control cultures lacking both ergosterol and oleic acid were incubated with each batch of experimental cultures. When growth in the control exceeded 0.05 mg dry wt ml^{-1} experimental cultures were discarded.

PREPARATION OF SPHEROPLASTS

Spheroplasts were prepared by a modification of the methods of Alterthum and Rose (1973) and Cartledge *et al.* (1977).

Anaerobically grown cultures of Sacch. cerevisiae Y185 were washed twice in buffered sorbitol (range 1.0 - 1.6 M containing, 20 mM-Tris and 10 mM-$MgCl_2$, adjusted to pH 7.2 with HCl) and resuspended in the same buffer to 10 mg dry wt ml^{-1}. After supplementation with Zymolyase 60000 [0.1 mg (6 mg dry wt organisms)$^{-1}$]or Zymolyase 100 000 [0.1 mg (10 mg dry wt organisms)$^{-1}$], the suspension was incubated at 30°C with orbital shaking (200 r.p.m.). Spheroplast formation was followed by diluting 0.1 ml portions of the suspension into 2.9 ml of either buffered sorbitol or water and measuring the optical density at 600 nm. Spheroplast formation was judged to be complete after 1 h (Pringle et al., 1979). A haemocytometer (New Improved Neubaur Haemocytometer; Weber, England) was used to count cells and spheroplasts before and after spheroplast formation respectively.

ESTIMATION OF OLEIC ACID INCORPORATION BY ORGANISMS

Saccharomyces cerevisiae Y185 was grown anaerobically in the presence of radioactively labelled [1-^{14}C]oleic acid. Prior to harvesting, five 3 ml samples of culture were removed and organisms harvested by centrifugation (2000 g; 10 min) using an MSE Centaur 2 centrifuge. Organisms were washed twice with water, resuspended in 0.5 ml 2 : 1 (v/v) chloroform/methanol, and transferred to scintillation vials containing 7.5 ml OptiPhase 'Safe' scintillant. The remaining organisms were harvested and converted into spheroplasts as previously described using 1.5 M buffered sorbitol. Prior to addition of Zymolyase, five 0.5 ml samples from the cell suspension were transferred to scintillation vials. After

spheroplasts were obtained, they were harvested by centrifugation (3000 g; 1 min; 4°C) and washed gently three times in buffered sorbitol to remove digested cell wall. Washed spheroplasts were resuspended in 1.5 M buffered sorbitol (25 ml) and five 0.5 ml samples were transferred to scintillation vials. Radioactivity was assayed using an LKB 1217 Rackbeta liquid scintillation spectrometer.

FRACTIONATION OF SPHEROPLAST LYSATES

Anaerobically grown cultures of Sacch. cerevisiae Y185 were supplemented with [1-^{14}C]oleic acid either at the start of growth or at various times before harvesting. Separation of cell organelles was achieved using a modification of the method of Henschke et al. (1983). Washed spheroplasts from 240 - 260 mg dry wt organisms were resuspended in 10 ml buffered mannitol (0.3 M, containing 50 mM-Tris, adjusted to pH 7.2 with HCl). Lysis of spheroplasts was effected by a combination of osmotic lysis and gentle mechanical disruption by incubating the spheroplast suspension for at least 20 min in an ice-water mixture and then subjecting the suspension to ten strokes of a Teflon-glass hand homogenizer (0.1 mm clearance; Jencons Ltd., Leighton Buzzard, England). A 10 ml portion of spheroplast lysate was layered on top of a discontinuous sucrose-density gradient consisting of 5 ml each of: 60, 50, 45, 40, 35, 30, 25, 20, 15 and 10% (w/v) sucrose. After centrifugation (50000 g; 150 min; 4°C) in an MSE Europa 75M ultracentrifuge (MSE Scientific Instruments, Crawley, Sussex, England) fitted with a 3 x 70 ml swing-out rotor, 20 fractions (3 ml) were collected using a peristaltic pump. A portion

(0.5 ml) of each fraction was assayed for radioactivity, and sucrose concentrations were measured with an Atago Hand Refractometer (Type 500; Atago Co., Ltd., Japan). Low-density vesicles and plasma-membrane fractions were characterized by transmission electron microscopy, and plasma membranes were further identified by a marker enzyme.

CHARACTERIZATION OF SUBCELLULAR ORGANELLES BY TRANSMISSION ELECTRON MICROSCOPY

Preparation of samples for transmission electron microscopy was done using a modification of the method of Henschke et al. (1983). Vesicles were fixed by mixing in a glass conical centrifuge tube with an equal volume of 4% (w/v) osmium tetroxide, and leaving for 1 h at 4°C with occasional agitation. Plasma-membrane preparations were first washed with an equal volume of 50 mM-sodium cacodylate and centrifuged in glass conical centrifuge tubes (2000 g; 10 min) using an MSE Centaur 2 centrifuge. Pellets were resuspended with 2% (w/v) osmium tetroxide in 50 mM-sodium cacodylate and incubated as described for vesicles. Samples were centrifuged as described above, pellets washed three times and resuspended in 2% (w/v) agarose. Suspensions were taken into Pasteur pipettes and extruded as they set. The solid agarose containing organelles was cut into small sections. Vesicle samples were stained with 1% (w/v) uranyl acetate at 20°C for 1 h in the dark. All samples were then dehydrated in the following acetone (v/v) solutions: 15% (1 x 10 min), 50% (1 x 10 min), 90% (1 x 10 min) and 100% (3 x 15 min). Preparations were transferred into Taab EM resin (Taab Laboratories Equipment, Reading, Berks, England), rotated for 24 h,

placed in Taab capsules and additional resin added before they were polymerized for 72 h at 60°C. Gold-silver sections were cut on a Reichert OMU3 ultramicrotome (Reichert-Jung, Slough, England) and stained for 10 min in ethanol (70%, v/v) saturated with uranyl acetate followed by 15 min in lead citrate (Reynolds, 1963). Sections were examined in a Zeiss EM109 transmission electron microsope (Carl Zeiss, Oberkochen, West Germany). All electron micrographs were taken on Ilford FP4 120 black and white roll film.

ENZYMIC CHARACTERIZATION OF PLASMA-MEMBRANE PREPARATIONS

A modification of the method of Dufour and Goffeau (1978) was used to solubilize proteins from subcellular organelles. Fractions (3 ml) were removed from discontinuous sucrose-density gradients as previously described, diluted in solubilization buffer (25 ml; 10 mM-Tris, containing 1 mM-ATP and 1 mM-EDTA, adjusted to pH 7.0 with HCl) and the suspension centrifuged (45000 g; 30 min; 4°C). The pellet was resuspended in solubilizing buffer containing lysophosphatidylcholine (2 mg ml^{-1}) , sonicated (2 x 30 sec; 3 mm probe; drive setting 7) using an MSE 100 W ultrasonicator and incubated for 20 min at 4°C. Following incubation, the suspension was centrifuged (11500 g; 30 min) in an MSE Micro Centaur and the supernatant retained for assay.

Adenosine triphosphatase activity was assayed by following release of Pi from ATP. The reaction mixture consisted of 1 ml 100 mM-Mes-Tris buffer (pH 6.5) containing 80 mM-KCl, 6 mM-$MgCl_2$ and solubilized sample (25 - 50 µg protein). Where indicated,

sodium orthovanadate (100 μM) was included specifically to inhibit plasma-membrane ATPase activity. The reaction was started by adding 6 mM-Na-ATP and the mixture incubated for 30 - 40 min at 30°C. The amount of Pi liberated was determined as described by Serrano (1978). The reaction was stopped by addition of 2 ml acidified molybdate solution (2.0%, v/v, conc. H_2SO_4 containing 0.5%, w/v, ammonium molybdate and 0.5%, w/v, SDS). Ascorbic acid (0.02 ml; 10% w/v) was added and the colour allowed to develop over 5 min at 30°C. Absorbance values (A_{750}) were related to Pi concentration by a calibration curve. Adenosine triphosphatase activities are quoted as μmol Pi liberated (mg protein)$^{-1}$min^{-1}.

The protein content of solubilized fractions was assayed using the Bio-Rad protein assay (Bio-Rad Laboratories, Munich, West Germany), a method based on the dye-binding technique of Bradford (1976). Portions (0.1 ml) of solubilized suspensions, diluted if necessary, were mixed with Bio-Rad dye reagent (5 ml; 20%, v/v, in water), the solution vortexed and the colour allowed to develop for 5 min at room temperature. Absorbance values (A_{595}) were related to protein content by a calibration curve prepared using bovine serum albumen as a standard.

LIPID EXTRACTION

Whole Organisms

Before harvesting organisms, a 2 ml solution containing 10 mg each of cycloheximide and chloramphenicol, and where indicated 10 ml 50 mM-para-chloromercuribenzoate (pCMB) in methanol, were injected into the culture which was incubated for a further 15 min.

Lipids were extracted from washed organisms by a modification of the Folch *et al.* (1957) procedure. Washed organisms were resuspended in 20 ml methanol and shaken in a Braun homogenizer (B. Braun, Melsungen, West Germany) for four periods of 30 sec at speed 2 (4000 r.p.m.) with glass beads (40 g; Sigma type V; 0.45 - 0.50 mm diam.). The sample bottle was cooled with expanding carbon dioxide during homogenization. Chloroform was added to the suspension to give 2 : 1 (v/v) chloroform/methanol, and the mixture stirred magnetically on a flat-bed stirrer for 2 h at room temperature (18 - 22°C). The suspension was filtered through Whatman no. 44 filter paper and the extraction procedure repeated on the residue. Extracts were pooled, washed with 0.25 vol. 0.88% (w/v) KCl and the mixture left to separate overnight at -20°C. The lower organic phase was removed, taken to dryness using a rotary evaporator, and the residue immediately dissolved in 1 ml light petroleum (b.p. 60 - 80°C), unless otherwise stated. Samples were stored under nitrogen gas at -20°C. Where indicated the extraction suspension was supplemented with 1.67 mM-*p*CMB.

Spheroplasts

Saccharomyces cerevisiae Y185 was grown anaerobically in the presence of [1-^{14}C]oleic acid and spheroplasts formed as previously described. Washed spheroplasts were resuspended in 20 ml methanol and chloroform added to give 2 : 1 (v/v) chloroform/methanol. Total lipid was extracted and stored as previously described.

Cell Organelles

Saccharomyces cerevisiae Y185 was grown anaerobically in the presence of [1-^{14}C]oleic acid and subcellular fractions collected as previously described.

Plasma membranes were suspended in 70 ml buffered mannitol (0.3 M, containing 50 mM-Tris, adjusted to pH 7.2 with HCl), and the suspension centrifuged (45000 g; 30 min; 4°C). The plasma-membrane pellet and the vesicles were each suspended in 10 ml methanol and chloroform added to give 2 : 1 (v/v) chloroform/methanol. Total lipids were extracted as previously described, except this time the suspensions were not filtered. After 2 h, an additional 30 ml 2 : 1 (v/v) chloroform/methanol was added to each suspension and lipids extracted for a further 2 h.

Culture Medium

Organisms from 500 ml culture fluid were removed by centrifugation (6000 g; 1 min; 4°C). Lipids were removed from the supernatant by shaking vigorously for 5 min after addition of ethyl acetate (3 x 100 ml). The suspension was allowed to separate and the top organic layer removed. Pooled extracts were taken to dryness and stored as previously described.

LIPID ANALYSIS

Analysis of Free and Esterified Long-Chain Alcohols

Lipid extracts were fractionated into lipid classes by chromatography on silicic acid columns prepared by a modification of the Naccarato et al. (1972) procedure. Graduated glass pipettes

(E-MIL; 5 ml) were plugged with glass wool and filled with 1 g SIL-LC silicic acid (325 mesh; Lipid Chromatography Grade; Hirsch and Ahrens, 1958). Maximal suction from a water aspirator, applied to the lower end of the column, caused slight compaction of the contents and assured even and reproducible packing. Packed columns were saturated with 20 ml light petroleum by overpressure with high-purity nitrogen gas. Lipid extracts were applied to the top of the column and eluted with 10 ml each of 4%, 7% and 10% (v/v) diethyl ether in light petroleum. A flow rate of 0.5 ml min^{-1} was maintained by nitrogen gas pressure on the column, and eluates were collected in 3 ml fractions. Fractions were taken to dryness under a stream of nitrogen gas, redissolved in an appropriate volume of light petroleum and 20 µl portions spotted onto a 20 x 20 cm 0.25 mm Silica Gel 60 precoated TLC plate (Merck). The plate was developed with a light petroleum (b.p. 40 - 60°C)-diethyl ether-acetic acid (70 : 30 : 1, by vol.) solvent mixture (Mangold, 1969), and lipids located by spraying with 0.2% (w/v) 2',7'-dichloro-fluoroscein in ethanol (Griffith et al., 1981) and viewed under ultraviolet (254 nm) radiation. Standards (1 mg ml^{-1} light petroleum (b.p. 60 - 80°C)) were cholesteryl palmitate, tripalmitin, stearic acid, palmitic acid, hexadecanol, octadecanol and ergosterol. To assay long-chain alcohols, appropriate fractions from the silicic acid column were pooled, concentrated under nitrogen gas and further separated by TLC using a solvent mixture of hexane-diethyl ether-acetic acid (30 : 70 : 1, by vol; Naccarato et al., 1972). Long-chain alcohols were visualized as previously described. The appropriate areas of the plate were scraped off and

lipids extracted using light petroleum-methanol-30% (w/v) NaCl (1 : 1 : 1, by vol.). Samples were shaken vigorously and allowed to separate. The top layer was removed and the the extraction procedure repeated twice. Extracts were pooled and taken to dryness under nitrogen gas.

Long-chain alcohols were separated and identified by GLC after conversion to their trimethylsilyl (TMS) ethers. Samples were taken up in 0.5 ml pyridine, and the solution mixed with an equal volume of bis-(trimethylsilyl) trifluoro-acetamide. The mixture was transferred to a 1 ml screw-top vial (Reacti-Vial; Pierce) and heated at 70°C for 15 min using a heated block (Techne, Dri-Block DB-3). Silylated samples were separated using Pye Unicam PU4500 capillary GLC fitted with a 25 m SE 30 capillary column. The injection temperature was 300°C, and the detector temperature 350°C. The initial column temperature was 190°C, and this was increased after 25 min at a rate of 16°C min^{-1} to give a final temperature of 250°C which was maintained for a further 10 min. The carrier gas (helium) flow rate was 1 ml min^{-1}, and the nitrogen gas flow rate, as a make-up gas across the detector, was 40 ml min^{-1}. Trimethylsilyl ethers of long-chain alcohols were identified by comparing their retention times with those of known standards and by co-chromatography with authentic standards. Peaks were analysed using a Pye Unicam CDPI computing integrator, and quantified by reference to an internal standard added during the extraction procedure after disruption of organisms. Identification of long-chain alcohols was verified by GLC-mass spectrometry, using electron-impact ionization or chemical ionization. Silylated

long-chain alcohols and fatty acids were used, and typical outputs of total ion-current versus time matched the GLC traces obtained with the Pye Unicam PU4500 capillary chromatograph previously described. The instrument used was a VG Analytical 70/70E, with a DB1 capillary column programmed at 150°C for 5 min and rising to 300°C at 30°C min^{-1} for 5 min and held at the upper temperature. The flow rate of the carrier gas (helium) was 1 ml min^{-1}, the resolution 1000, the electron impact 70 eV and the calibrated range 20 - 580.

To examine the effect of pCMB on extraction of esterified long-chain alcohols, certain fractions from the silicic acid column, which contained neutral lipids, were pooled and applied to Silica Gel 60 plates. Lipids were separated using the solvent system light petroleum-diethyl ether-acetic acid (90 : 10 : 1, by vol.; Kates, 1972), and visualized as already described. Lipid bands with mobilities similar to heptadecanyl acetate, the internal standard, were scraped off the plate, and lipids extracted with light petroleum as already described. Samples were transferred to a 5 ml Reacti-Vial and dried under a stream of nitrogen gas. Samples were saponified using a modification of the method of Pollard et al. (1979), the Reacti-Vial being supplemented with 5 ml M-KOH in 95% methanol, sealed and heated at 80°C for 3 h. After cooling, samples were diluted with 5 ml methanol, and non-saponifiable lipids removed with 3 x 5 ml light petroleum. They were then prepared for GLC and analysed as already described.

Analysis of Lipid Fatty-Acyl Composition

Lipid extracts from organisms were dissolved in chloroform to give approximately 25 mg lipid ml^{-1}. To prepare fatty-acid methyl esters of total lipids from organisms, 1 ml lipid solution was transferred to a 5 ml Reacti-Vial and the solution taken to dryness under a stream of nitrogen gas. Boron trifluoride (14%, w/v, in methanol; 3 ml) was added to the vial which was sealed and heated for 1 h in a dry block at 80°C. To extract fatty-acid methyl esters, the reaction mixture was made up to 5 ml with methanol and the solution supplemented with 5 ml each of light petroleum and 30% (w/v) NaCl. The solution was shaken vigorously and the mixture left to separate. Fatty-acid methyl esters in light petroleum were removed and the procedure repeated a further two times. The solution containing methyl esters was taken down to a small volume under a stream of nitrogen gas, streaked onto a 20 x 20 cm 0.25 mm Silica Gel 60 precoated TLC plate and the plate developed with a light petroleum-diethyl ether-formic acid (75 : 25 : 0.5, by vol.) solvent mixture (Fixter et al., 1986). Fatty-acid methyl esters were scraped off the plate, extracted with light petroleum as previously described, and taken down to a small volume under a stream of nitrogen gas. Fatty-acid methyl esters were analysed using a fused silica capillary column (25 m length; SGE BP 21) in a Pye Unicam GCD chromatograph fitted with an SGE on-column adaptor. The injection temperature was 240°C. The column was maintained at 135°C for the first 5 min, after which the temperature was raised at the rate of 8°C min^{-1} until it reached 180°C. The carrier gas was hydrogen. Percentage fatty-acyl compositions were calculated

using a Pye Unicam CDPI computing integrator. Individual lipid classes in total lipid extracts were separated by streaking a solution containing 10 mg lipid onto a 20 x 20 cm 0.25 mm Silica Gel 60 precoated TLC plate, which was developed with a solvent mixture containing light petroleum (b.p. 40 - 60°C)-diethyl ether-acetic acid (70 : 30 : 1, by vol.). Lipid classes were identified as already described, bands scraped off the plates, and fatty-acid methyl esters of the lipids prepared as described previously.

Analysis of Oleic Acid Incorporation into Lipid Classes

Individual lipid classes in total lipid extracts of whole organisms, spheroplasts and cell organelles from Sacch. cerevisiae Y185 grown anaerobically in the presence of [1-^{14}C]oleic acid, were separated by streaking lipid extracts onto a 20 x 20 cm 0.25 mm Silica Gel G precoated TLC plate (Whatman), which was then developed with a solvent mixture containing light petroleum-diethyl ether-acetic acid (70 : 30 : 1, by vol.). Lipid classes were identified as already described, cut out and transferred to scintillation vials. Radioactivity was assayed as previously described.

ASSAY OF LIPASE ACTIVITY

Candida albicans NCYC 1467 grown under self-induced anaerobic conditions for 120 h was used. Lipase activity was assayed using tributyrin or olive oil and measuring release of fatty acids directly by titration. An automatic titrator consisting of a Standard pH meter PHM 82, Titrator TTT80, Autoburette ABU 80,

Servograph REC80 and Titration Assembly TTA80 (Radiometer-Copenhagen) was used to control the pH at various values during the assay, and also to titrate any fatty acids liberated during hydrolysis.

An emulsion of 5% (w/v) tributyrin or olive oil in 2% (w/v) gum arabic was formed by sonication using an Ultrasonic Generator (Type 7533A; set to continuous for 3 - 4 min; drive setting 7). An aliquot of emulsion (15 ml), plus 0.35 ml 22% (w/v) $CaCl_2$ for the olive oil assay, was transferred to a Radiometer reaction vial and allowed to equilibrate at 30°C for 10 min in a water bath. Lipase activity was examined at various pH values after the addition of 1 ml of cell suspension or water. Where indicated, cultures were pre-incubated with 10 ml 50 mM-pCMB in methanol for 15 min. The reaction mixture was stirred vigorously throughout the assay and the titrator recorded the amount of 0.1 M-NaOH required to maintain the reaction mixture at the set pH value.

PRODUCTION OF LONG-CHAIN ALCOHOL BY CELL-FREE EXTRACTS

Preparation and Fractionation of Cell-Free Extracts

Candida albicans NCYC 1467 grown under self-induced anaerobic conditions for 72 h was used. Organisms (2 g dry wt) were washed twice with potassium phosphate buffer (100 mM, pH 6.5, containing 5 mM-2-mercaptoethanol), suspended in 10 ml buffer and the suspension shaken in a Braun homogenizer as already described. The homogenate was suspended in 120 ml buffer and whole organisms and debris removed by centrifugation (500 g; 5 min; 4°C). The supernatant was centrifuged at 18 000 g for 30 min at 4°C to remove

mitochondria and/or promitochondria. A microsomal fraction was obtained by centrifuging the post-mitochondrial supernatant (100 000 g; 90 min; 4°C) in an MSE Europa 75M Ultracentifuge fitted with a 3 x 70 ml swing-out rotor, and resuspended in 6 ml buffer. Protein in the supernatant obtained after removal of the microsomal fraction was precipitated as described by Naccarato et al. (1974). Ammonium sulphate (heavy metal-free) was added to the supernatant to give 55% saturation, the suspension stirred on a magnetic flat-bed stirrer for 15 min at 4°C, and the precipitated protein collected by centrifugation (12 000 g; 20 min; 4°C). The protein was redissolved in 6 ml buffer.

Protein determinations were performed as already described, except that microsomal fractions (0.1 ml) were first solubilized by boiling for 5 min after addition of an equal volume of 0.1 M-NaOH. The mixture was then neutralized with 0.1 M-HCl (0.1 ml) and 0.2 ml acetic acid-sodium acetate buffer (0.2 M; pH 5.0) added.

Assay of Hexadecanol Formation

Hexadecanol formation was assayed using [1-^{14}C]palmitoyl-CoA. The reaction mixture (final vol. 2 ml), in a 5 ml Reacti-Vial, consisted of 100 mM-potassium phosphate buffer (pH 6.5) containing 5 mM-2-mercaptoethanol, 2 mg bovine serum albumin (fatty acid-free), 0.3 μmol NADH or NADPH, 20 nmol [1-^{14}C]palmitoyl-CoA [58 mCi (2.15 GBq) mmol^{-1}] and 2 mg microsomal or soluble protein. The reaction mixture was incubated for 1 h at 30°C on an orbital shaker (200 r.p.m.) after the head space had been flushed with nitrogen gas. The reaction was terminated by adding 3 ml methanol,

and lipids extracted by adjusting the solvent concentration to 1 : 1 : 2 (by vol.) water-methanol-chloroform. Hexadecanol was added, as carrier, to the extraction solvent such that 25 nmol was present in each reaction mixture. The lower organic phase was removed, concentrated under a stream of nitrogen gas and spotted onto a 20 x 20 cm 0.25 mm Silica Gel 60 precoated TLC plate. The plate was developed using a hexane-diethyl ether-acetic acid (30 : 70 : 1, by vol.) solvent mixture, and long-chain alcohols removed and prepared for GLC analysis as already described. A 1 µl portion was analysed by quantitative GLC to determine the extent of carrier recovery, estimated from the ratio of the hexadecanol peak area in the sample to the peak area of hexadecanol present in the extraction solvent. Radioactivity in the remaining sample was assayed as previously described, and the amount of hexadecanol synthesized was calculated by dividing the corrected radioactivity by the specific activity of the sample substrate.

MATERIALS

All chemicals used were AnalaR grade or of the highest purity available commercially. Adenosine 5'-triphosphate (disodium salt), boron trifluoride, bovine serum albumin, 2',7'-dichlorofluorscein pCMB, bis-(trimethylsilyl) trifluoro-acetamide, reduced nicotinamide adenine dinucleotide (disodium salt), reduced nicotinamide adenine dinucleotide phosphate (tetrasodium salt), silicic acid and all lipids and lipid standards were purchased from Sigma Chemical Co. Ltd., Poole, Dorset, England. Cycloheximide,

chloramphenicol and sodium orthovanadate were obtained from BDH Chemicals Ltd., Poole, Dorset, England. Zymolyase was provided by ICN Biomedicals Ltd., High Wycombe, Bucks, England. Radioactively labelled oleic acid and palmitoyl-CoA were purchased from Amersham International, Amersham, England. OptiPhase 'Safe' scintillation fluid was obtained from Fisons plc, Scientific Equipment Division, Loughborough, England. Gas-liquid chromatography columns were provided by SGE Scientific Glass Engineering Ltd., Milton Keynes, England.

RESULTS

OLEIC ACID INCORPORATION INTO SACCHAROMYCES CEREVISIAE Y185

Growth of Saccharomyces cerevisiae Y185 Under Anaerobic Conditions

Saccharomyces cerevisiae Y185, grown anaerobically in the presence of oleic acid and ergosterol, entered the exponential phase of growth after approximately 8 h and approached stationary phase of growth after about 18-20 h incubation (Fig. 2). The generation time was 1 h 45 min and organisms reached the mid exponential-phase of growth (0.24-0.26 mg dry wt ml^{-1}) after approximately 13 h 30 min. The final growth yield approximated 1.0 mg dry wt ml^{-1}.

Formation and Recovery of Spheroplasts from Anaerobically Grown Saccharomyces cerevisiae Y185

The concentration of buffered sorbitol used as an osmotic stabilizer during formation of spheroplasts from anaerobically grown Sacch. cerevisiae Y185 had a marked effect on their recovery (Fig. 3). At concentrations between 1.0 and 1.2 M, the recovery was approximately 50%. At a concentration of 1.4 M-buffered sorbitol there was a mean recovery of about 80%, but this was highly variable. Microscopic examination of spheroplasts produced in 1.6 M-buffered sorbitol revealed slight shrinkage, suggesting that higher concentrations would not offer any more protection against lysis. For further studies requiring the use of spheroplasts, 1.5 M-buffered sorbitol was used giving a recovery of approximately 80%.

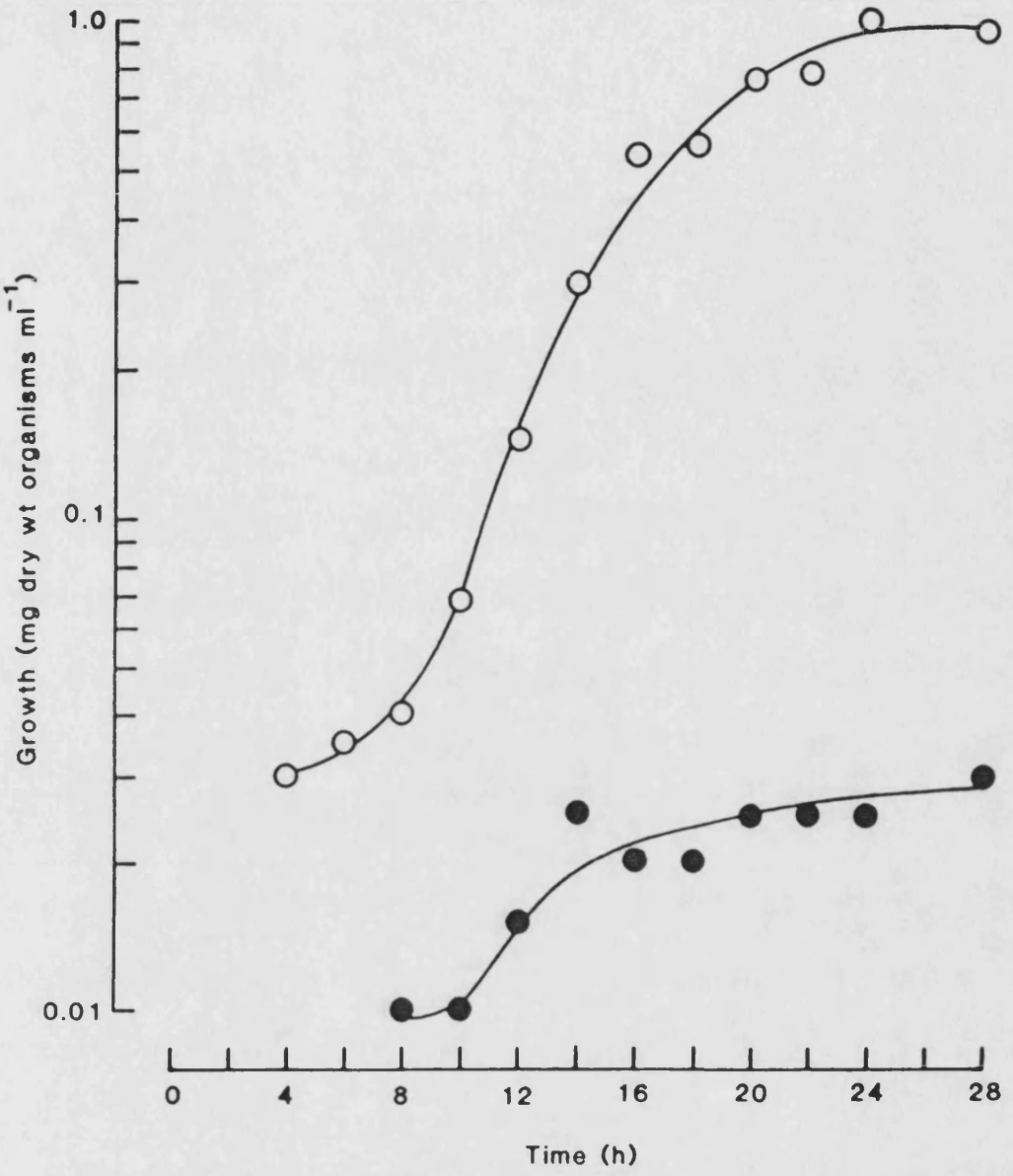


Figure 2. Time-course of anaerobic growth of Saccharomyces cerevisiae Y185 in medium supplemented with oleic acid and ergosterol (O) and in unsupplemented medium (●). Values plotted are the means of two independent determinations.

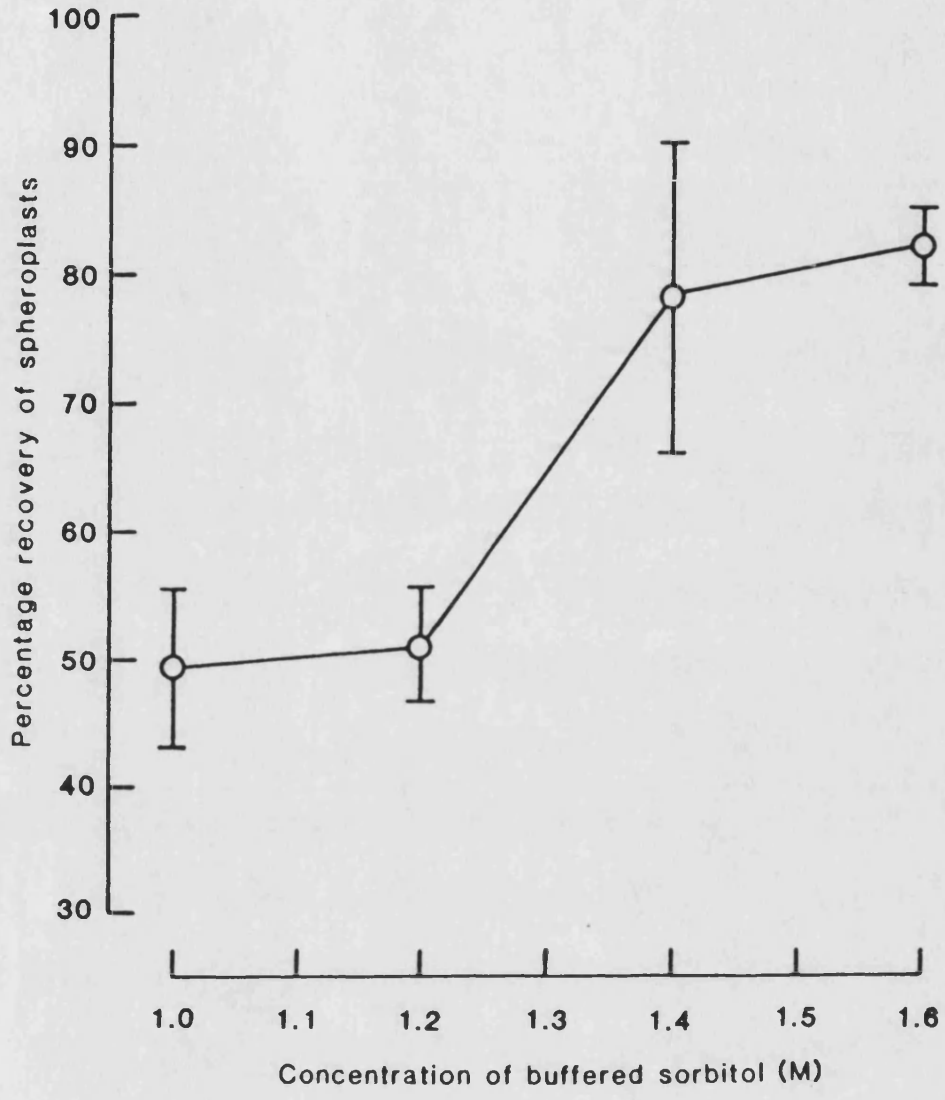


Figure 3. Recovery of spheroplasts from anaerobically grown Saccharomyces cerevisiae Y185 using different concentrations of buffered sorbitol. Values plotted are the means of three independent determinations ± SD.

Estimation of Oleic Acid Incorporation by Anaerobically Grown Saccharomyces cerevisiae Y185

By growing Sacch. cerevisiae Y185 in the presence of [1-^{14}C] oleic acid, it was possible to estimate the amount of oleic acid incorporated into whole organisms and intact spheroplasts (Table 1). From 250 mg dry wt cells, 49.6% of the 30 mg oleic acid supplement appeared to be associated with whole cells. However, when the cell walls were removed, 45.5% (having taken into account losses during formation of spheroplasts) was shown to have been incorporated into the spheroplasts. Recovery of [1-^{14}C]oleic acid activity ranged between 78 and 93%, presumably due to unavoidable non-specific binding.

Fractionation of Spheroplast Lysates from Anaerobically Grown Saccharomyces cerevisiae Y185 and Location of Incorporated Oleic Acid

Separation and identification of subcellular fractions

Spheroplast lysates from anaerobically grown Sacch. cerevisiae Y185 produced two distinct regions of turbidity in the discontinuous sucrose-density gradient. One very dense single band at the top of the gradient corresponded to a sucrose density of 1.035 g ml^{-1}, while the other was made up of three closely associated dense bands starting approximately half way down the gradient and corresponded to sucrose densities between 1.130 and 1.190 g ml^{-1} (Fig. 4). These bands were labelled A, B, C and D respectively.

Table 1. Estimation of oleic acid incorporated into anaerobically grown Saccharomyces cerevisiae Y185 harvested from mid exponential-phase cultures. Values quoted are the means of five independent determinations ± SD.

	Contents of oleic acid in: Whole cells	Spheroplasts
Percentage (%)	49.6 ± 5.7	45.5 ± 5.3
Quantity (mg)	14.9 ± 1.7	13.7 ± 1.6

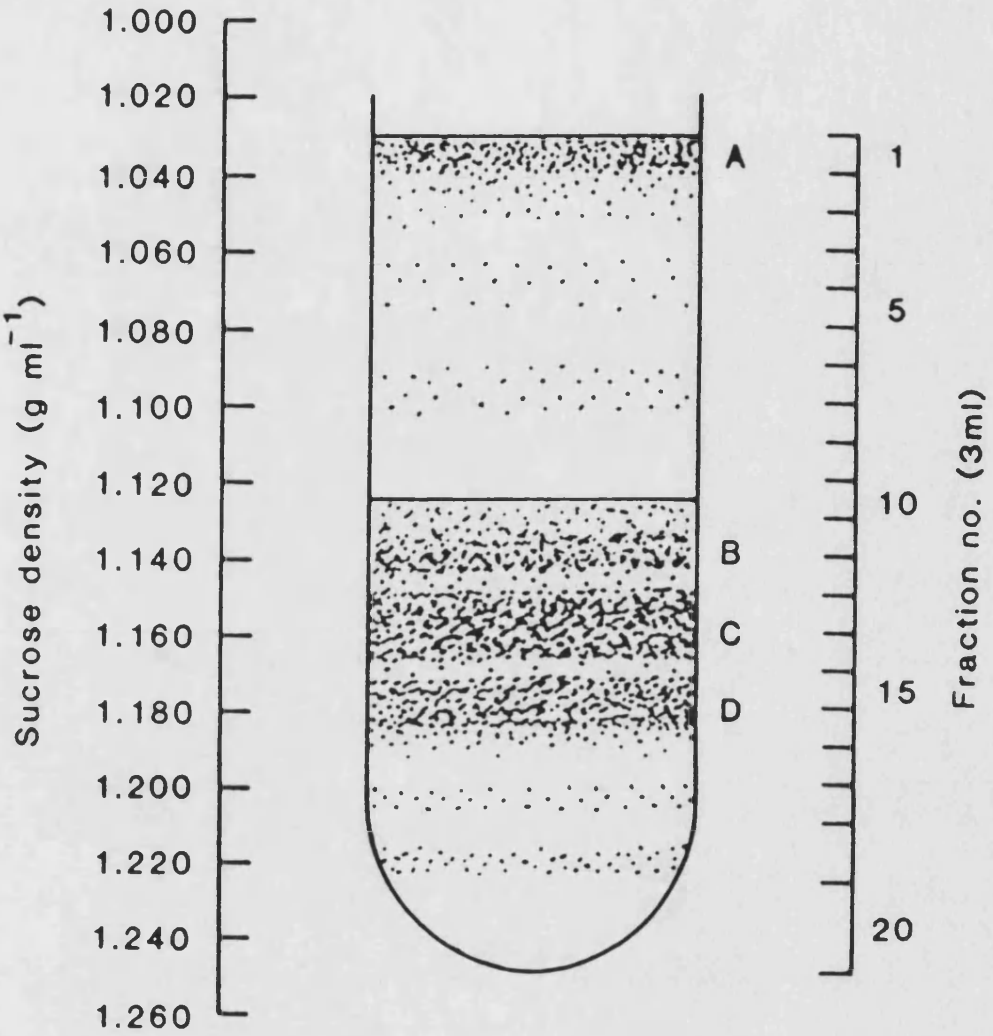


Figure 4. Drawing of a discontinuous sucrose-density gradient showing separation of cellular organelles from a spheroplast lysate of anaerobically grown Saccharomyces cerevisiae Y185. A, B, C and D represent major bands.

When band A was examined by transmission electron microscopy, it was found to contain many vesicles ranging in diameter from 0.25 to 0.50 μm (Plate 1). Transmission electron microscopic examination of bands B, C and D revealed the presence of plasma membranes (Plates 2-5). What appeared to be associated with these plasma-membrane preparations, to varying degrees, were vesicles which may account for the separation into three closely associated bands. Closer examination of the plasma membranes from band B suggested that the vesicles were not artefacts of spheroplast lysis or contamination from other subcellular organelles. They appeared to form an integral part of the plasma membrane and ranged in size from less than 0.10 to 0.25 μm diameter (Plate 3).

Further characterization of bands B, C and D was achieved using plasma-membrane ATPase as a marker enzyme (Fig. 5). A peak for protein content and plasma-membrane ATPase was found in the discontinuous sucrose-density gradient at densities corresponding to bands B, C and D (1.130-1.190 g ml^{-1}). Plasma-membrane ATPase was completely inhibited by pre-incubation of the membrane preparation with orthovanadate (100 μM). The protein content of band A did not have a corresponding peak for plasma-membrane ATPase activity.

Subcellular location of oleic acid

Fractionated spheroplast lysates from Sacch. cerevisiae Y185 grown anaerobically in the presence of [1-^{14}C]oleic acid showed significant incorporation (greater than 5%) of oleic acid into bands A, B, C and D (Fig. 6). When [1-^{14}C]oleic acid was added to

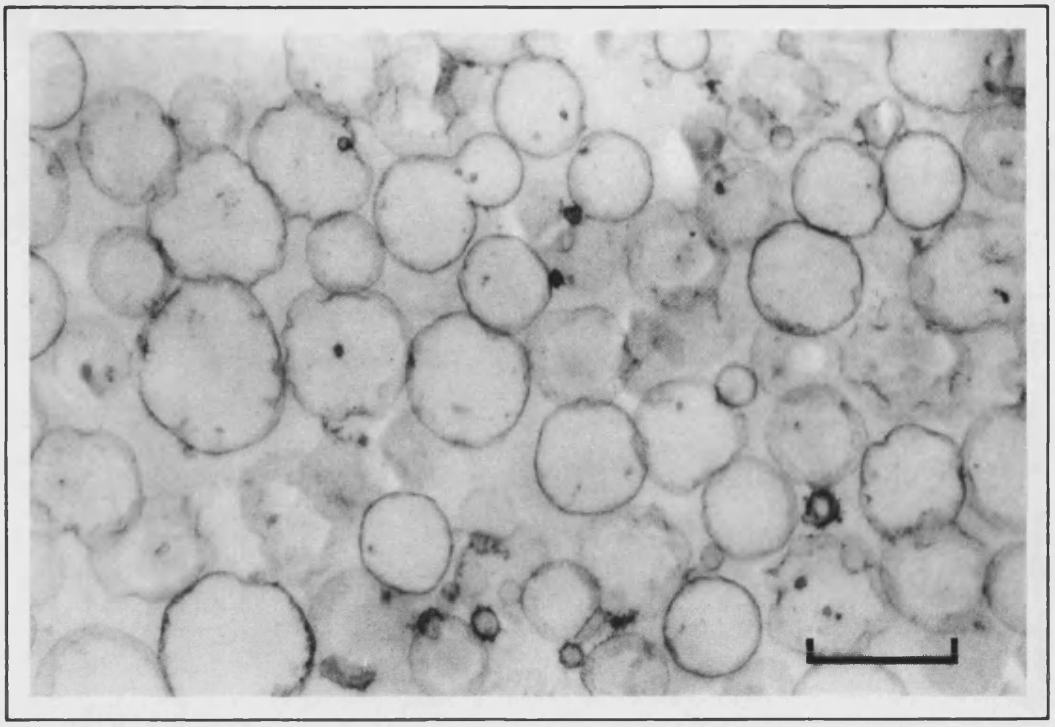
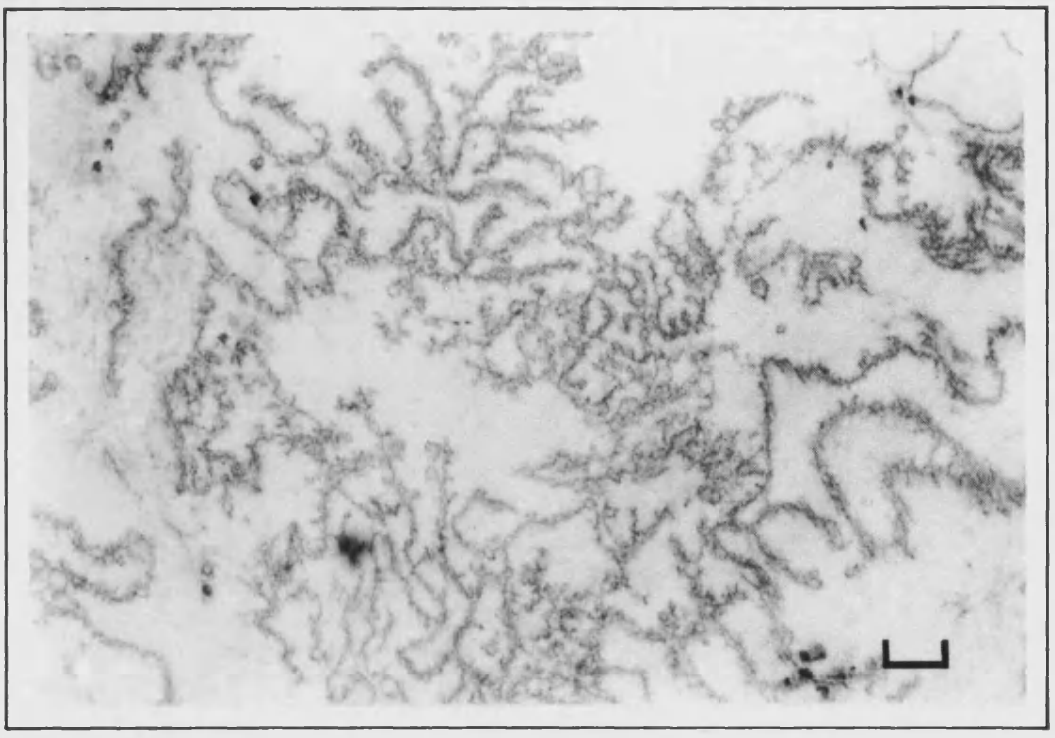


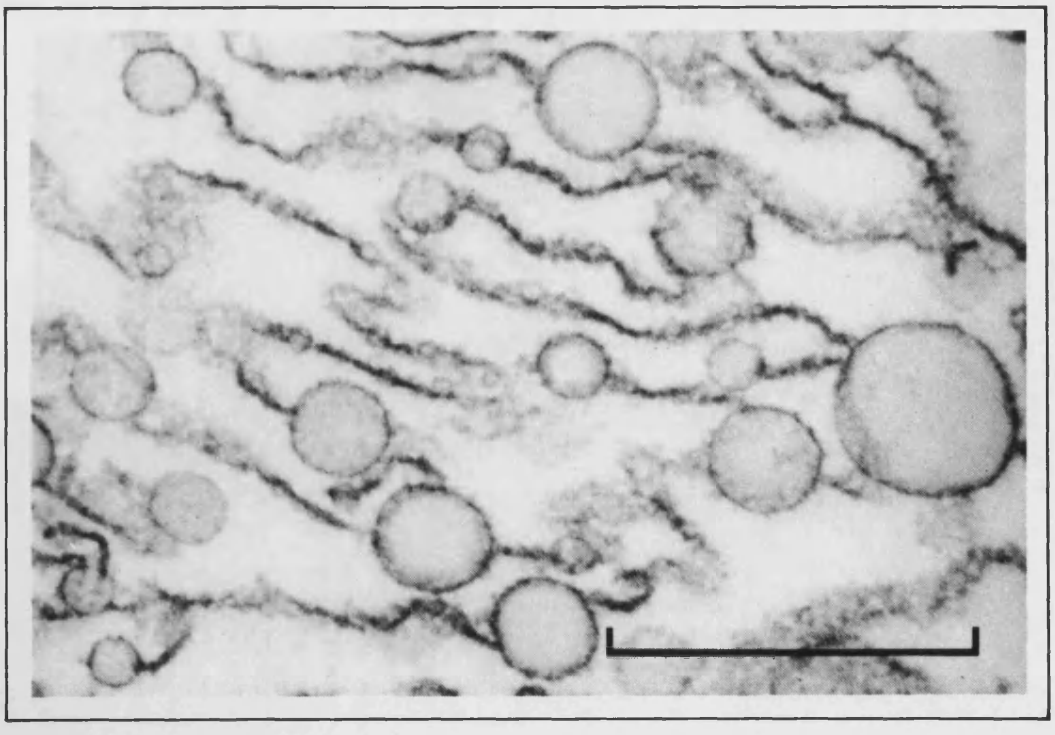
Plate 1. Transmission electron micrograph of band A isolated from a spheroplast lysate of anaerobically grown Saccharomyces cerevisiae Y185 (see Fig. 4).

This micrograph clearly shows the presence of relatively uncontaminated low-density vesicles ranging in diameter from 0.25 to 0.50 µm. Scale bar represents 0.5 µm.

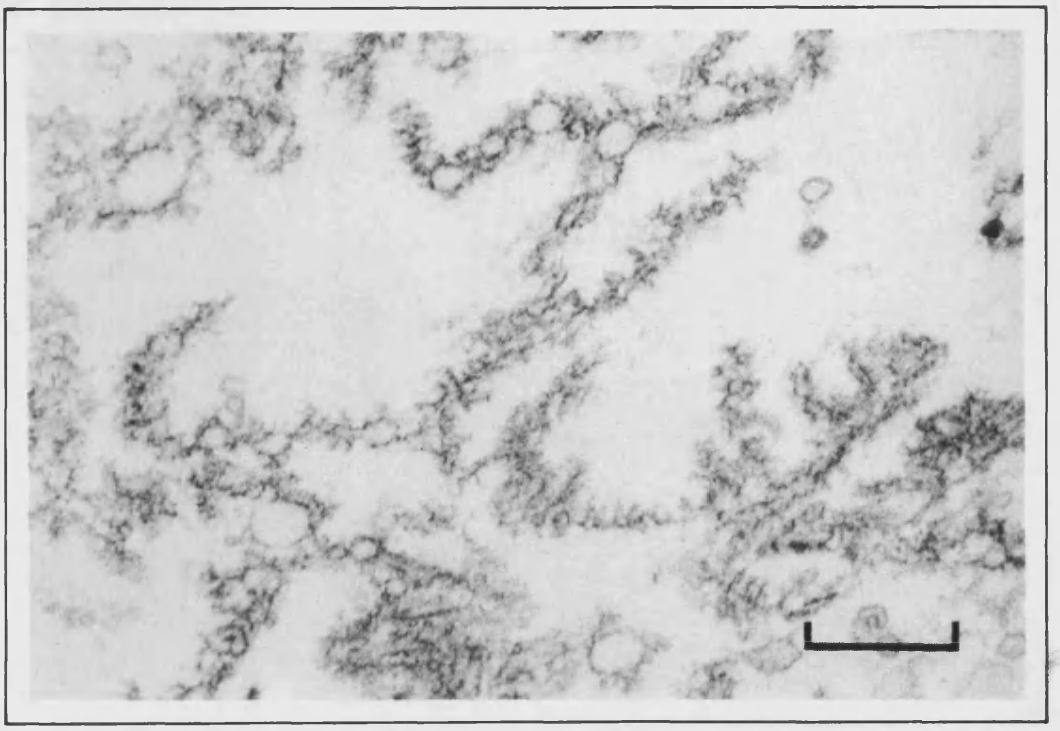
Plate 2. Transmission electron micrograph of band B isolated from a spheroplast lysate of anaerobically grown Saccharomyces cerevisiae Y185 (see Fig. 4), showing the presence of plasma membranes. Also associated with plasma membranes is a high proportion of vesicles. Scale bar represents 0.5 μm.

Plate 3. Higher magnification transmission electron micrograph of band B showing vesicular associations with plasma membranes. Vesicle diameters range from less than 0.10 to 0.25 μm. Scale bar represents 0.5 μm.





Plates 4 and 5. Transmission electron micrographs of bands C and D isolated from a spheroplast lysate of anaerobically grown Saccharomyces cerevisiae Y185 (see Fig. 4). Micrographs show plasma membranes with varying degrees of vesicular associations. Scale bars represent 0.5 μm.



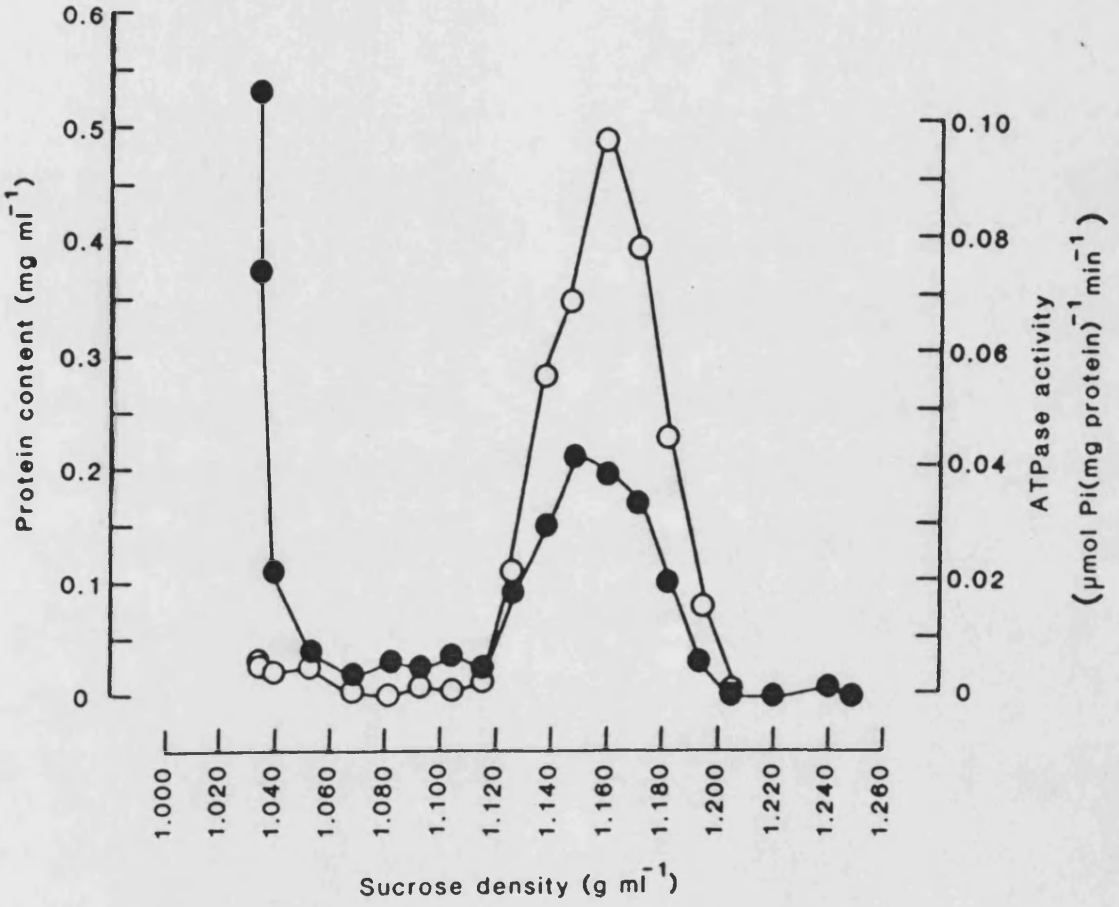
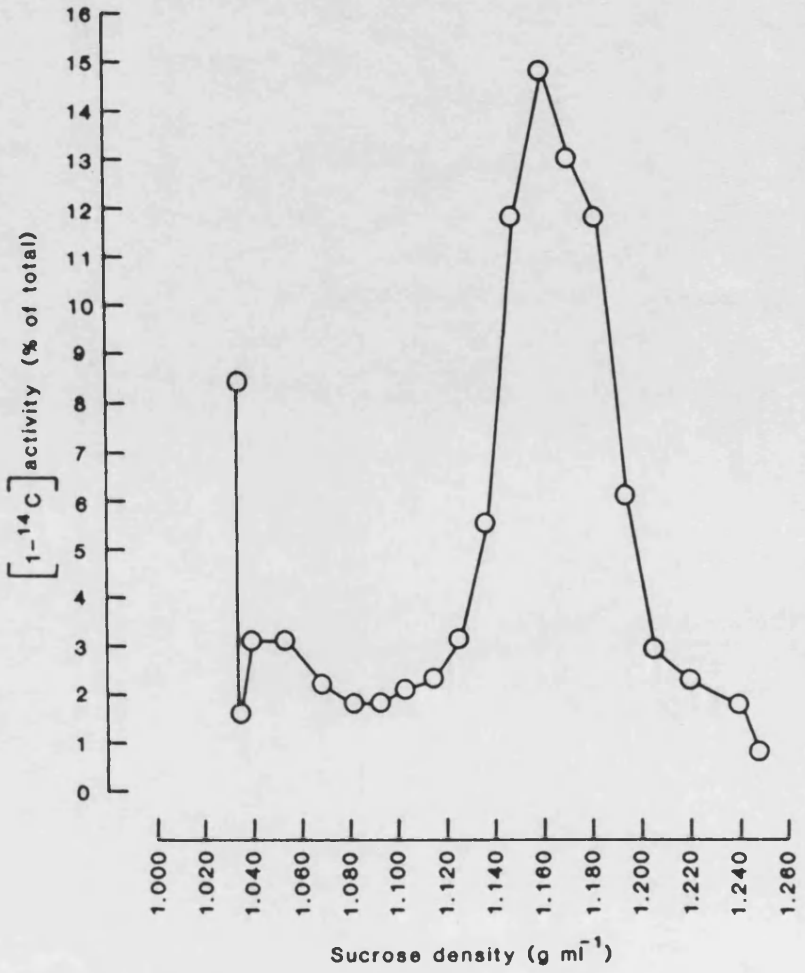
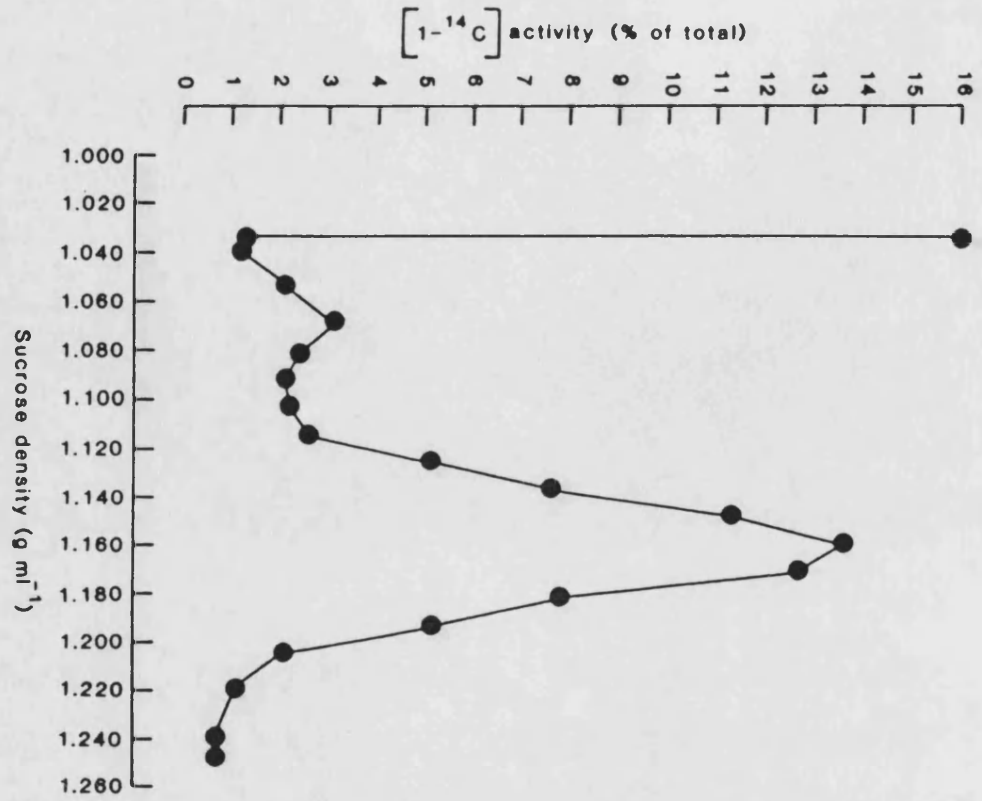
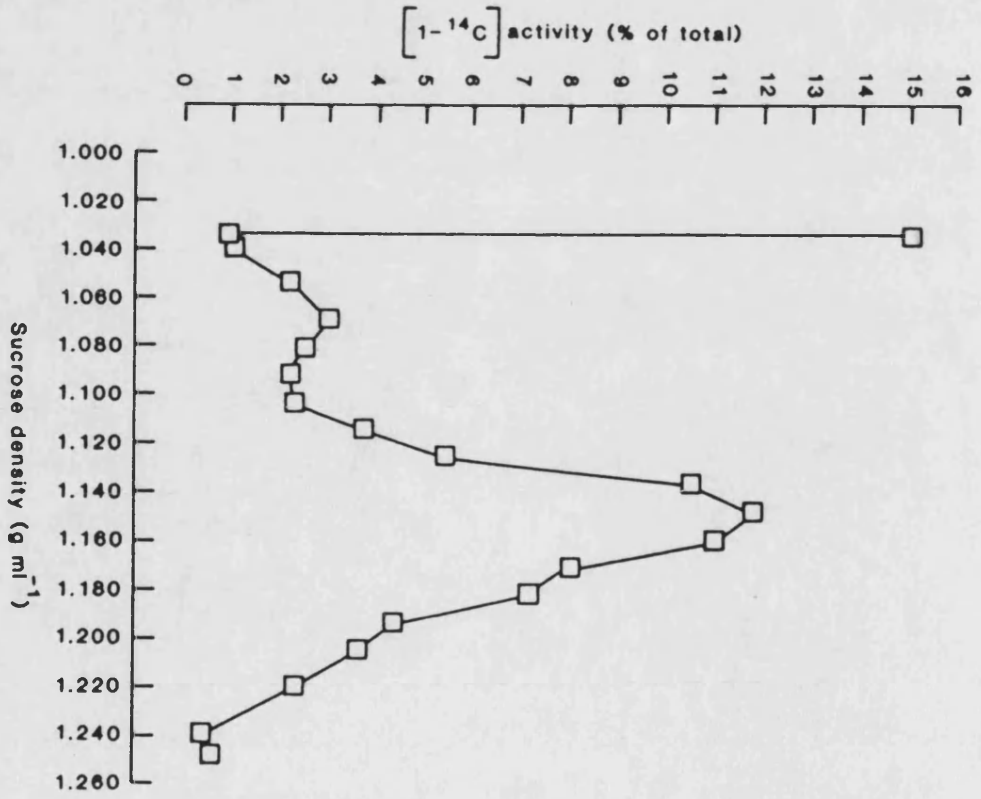


Figure 5. Distribution of plasma-membrane ATPase activity (O) and protein content (●) through a fractionated spheroplast lysate of anaerobically grown Saccharomyces cerevisiae Y185.
Values plotted are the means of two independent determinations.

Figure 6. Representative plots of the distribution of [1-^{14}C]oleic acid through a fractionated spheroplast lysate of anaerobically grown Saccharomyces cerevisiae Y185. ○ indicates that [1-^{14}C]oleic acid was present at inoculation, ● that it was added to the culture one doubling time before harvesting, and □ that it was added to the culture 0.5 doubling time before harvesting.




[1-14C] activity (% of total)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Sucrose density (g ml-1)
1.000
1.020
1.040
1.060
1.080
1.100
1.120
1.140
1.160
1.180
1.200
1.220
1.240
1.260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1.120
1.140
1.160
1.180
1.200
1.220
1.240
1.260

the cultures one doubling- and 0.5 doubling-time before harvesting, the radioactivity profiles through the discontinuous sucrose-density gradients were essentially the same (Fig. 6), with the exception that the relative amount of [1-^{14}C]activity in band A had doubled. Table 2 shows that an increased activity in the low-density vesicles (band A) leads to a corresponding decrease in [1-^{14}C]activity in the plasma membrane bands B, C and D.

Incorporation of oleic acid into various classes of lipid

Lipid extracts from whole cells of Sacch. cerevisiae Y185 grown anaerobically in the presence of [1-^{14}C]oleic acid and harvested from mid exponential-phase cultures contained a high proportion of free [1-^{14}C]oleic acid. Phospholipids contained 43.7% of the incorporated oleic acid, while only 8.8 and 0.3% was incorporated into the triacylglycerol and sterol-ester fractions, respectively (Table 3). When organisms were converted into spheroplasts over a period of 1 h, the distribution of [1-^{14}C]oleic acid activity changed drastically. Most notable was the transfer of label from free fatty acids to the triacylglycerol and sterol-ester fractions. There also appeared to be some deacylation of the phospholipids (Table 3).

Isolated plasma membranes had a high proportion of [1-^{14}C]oleic acid associated with the triacylglycerol and sterol-ester fractions, amounting to approximately 40%. This could possibly be explained by vesicular association with the plasma membranes (Plates 2-5) and is supported by the presence of an extremely high proportion of [1-^{14}C]oleic acid in the triacylglycerol fraction and

Table 2. Relative distribution of [1-^{14}C]oleic acid activity in low-density vesicles and plasma membranes from anaerobically grown Saccharomyces cerevisiae Y185. Values quoted are the means of four independent determinations ± SD.

Time of [1-^{14}C]oleic acid supplementation before harvesting (h)	Contents of [1-^{14}C]oleic acid (percentage of total incorporated into spheroplasts) in:	
	Vesicles (Band A)	Plasma membranes (Bands B, C and D)
13.50 (start)	7.5 ± 1.8	65.0 ± 2.5
1.75 (1 doubling)	16.0 ± 1.0	56.0 ± 4.8
0.88 (0.5 doubling)	14.4 ± 0.7	57.2 ± 1.4

Table 3. Incorporation of oleic acid into lipid classes isolated from cells and cellular fractions of anaerobically grown Saccharomyces cerevisiae Y185. Values quoted are the means of three independent determinations ± SD. nd indicates that the lipid class was not detected.

Cellular fraction	Contents of [1-^{14}C]oleic acid (percentage of total) in:				
	Phospholipids	Free fatty acids	Diacylglycerols	Triacylglycerols	Sterol esters
Whole cells	43.7 ± 1.3	42.9 ± 2.3	4.2 ± 0.1	8.8 ± 1.8	0.3 ± 0.1
Spheroplasts	31.3 ± 1.6	14.1 ± 1.0	2.1 ± 0.3	49.4 ± 2.1	3.1 ± 0.2
Plasma membranes	40.7 ± 1.3	17.3 ± 1.0	3.3 ± 0.7	35.4 ± 1.2	3.4 ± 0.4
Low-density vesicles	3.8 ± 0.9	4.0 ± 1.0	nd	85.3 ± 0.2	7.0 ± 1.4
Plasma membranes + low-density vesicles	29.0 ± 2.4	13.1 ± 0.5	2.2 ± 0.6	51.2 ± 2.9	4.5 ± 0.6

the raised proportion in the sterol-ester fraction of the low-density vesicles (Table 3). Combining the [1-^{14}C]oleic acid activities of lipid classes isolated from plasma membranes and low-density vesicles led to a relative distribution of [1-^{14}C]activity similar to that of intact spheroplasts (Table 3).

LONG-CHAIN ALCOHOL PRODUCTION BY YEASTS

Identification of Long-Chain Alcohols

The mobility of certain lipid fractions on silicic-acid columns and TLC plates suggested that their polarity was that expected of long-chain alcohols (Fig. 7). Subsequent purification, separation and identification by GLC and GLC-mass spectrometry confirmed the presence of $C_{14:0}$, $C_{16:0}$ and $C_{18:0}$ primary alcohols in lipid extracts of organisms. Figure 8 shows the total ion-current trace from GLC-mass spectrometry of a purified lipid extract from C. albicans grown under self-induced anaerobic conditions. The peak at scan no. 253 was identified as 1-hexadecanol from its fragmentation pattern in electron-impact ionization (Fig. 9) and chemical ionization (Fig. 10) mass spectrometry. Peaks 36, 115 and 362 were generated by $C_{12:0}$, $C_{14:0}$ and $C_{18:0}$ long-chain alcohols, respectively (Fig. 8). Peak 309 was due to the 1-heptadecanol internal standard. Other components of the extract identified were members of the isoprenoid alcohol series (peaks 111, 128, 378 and 403). This latter group of compounds was found in all lipid extracts examined by TLC, and they were also identified in extracts of yeasts that lacked the ability to synthesize long-chain alcohols.

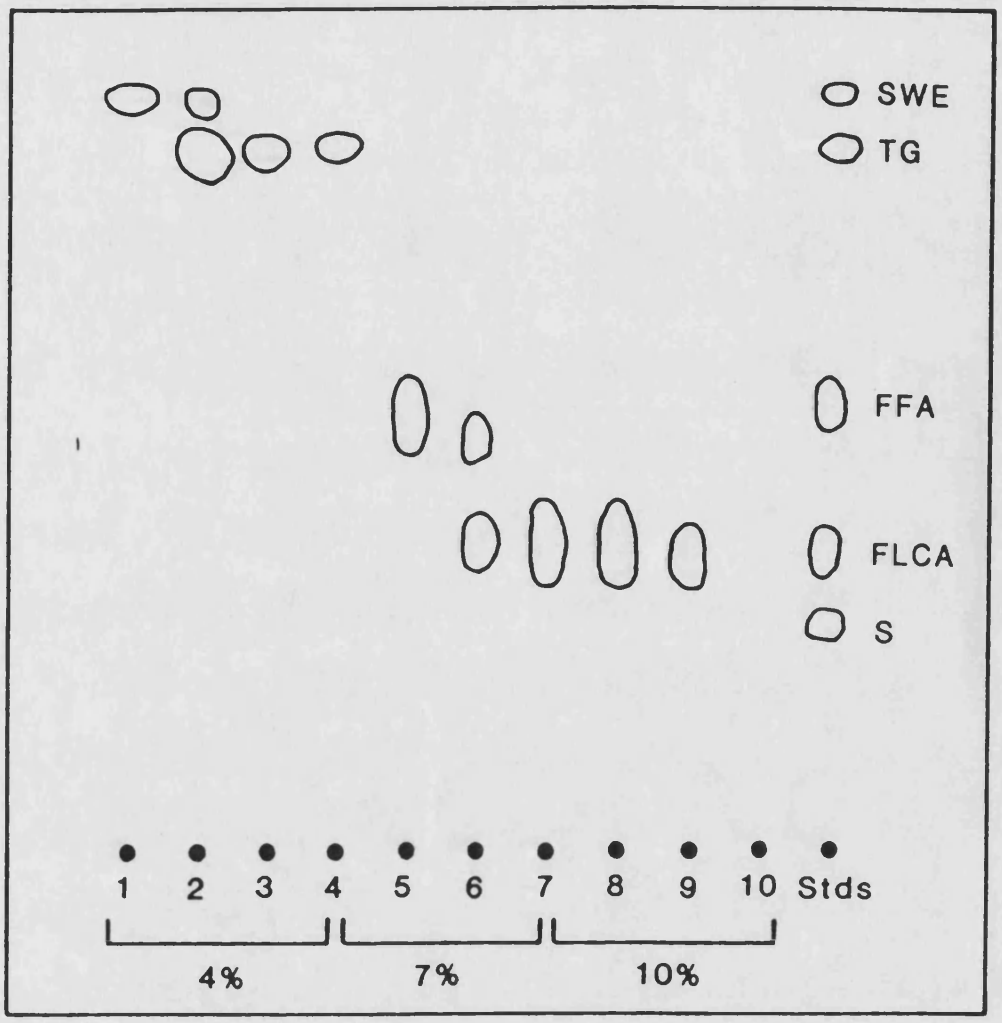


Figure 7. Drawing of a TLC plate showing separation of lipid classes in each of the 10 fractions (3 ml) obtained by silicic-acid column chromatography of a lipid extract of *Candida albicans* grown under self-induced anaerobic conditions for 72 h. 4%, 7% and 10% represents the diethyl ether/petroleum ether elution mixtures used in column chromatography. SWE indicates sterol/wax esters, TG triacylglycerols, FFA free fatty acids, FLCA free long-chain alcohols, S sterols and Stds lipid standards.

Figure 8. Total ion-current trace from GLC-mass spectrometry of a purified lipid extract from *Candida albicans* grown under self-induced anaerobic conditions for 168 h. Separation of individual alcohols resembled that obtained using a Pye Unicam PU 4500 capillary GLC (see text).

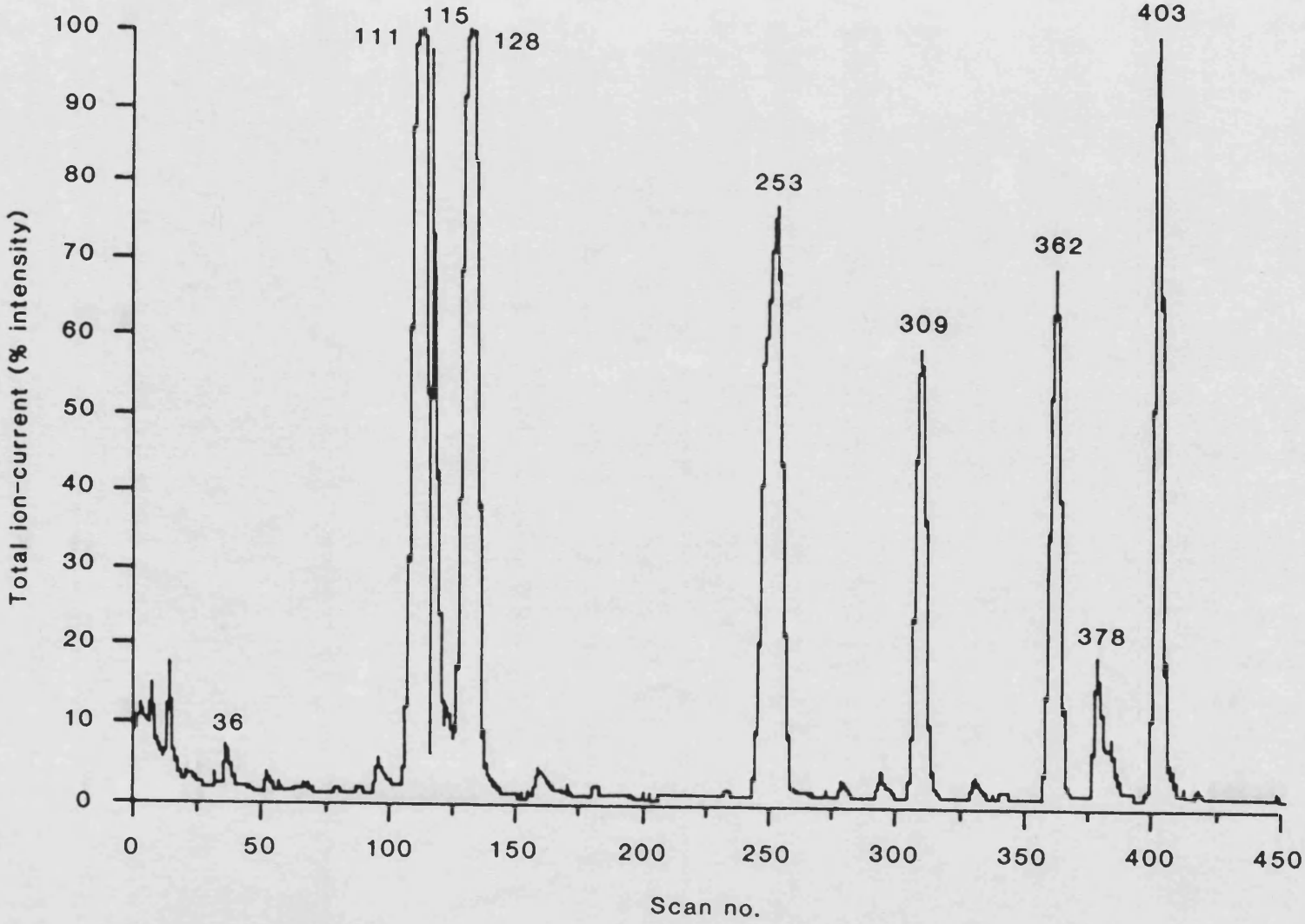

Total ion-current (% intensity)
100
90
80
70
60
50
40
30
20
10
0
111
115
128
253
309
362
378
403
36
0
50
100
150
200
250
300
350
400
450
Scan no.

Figure 9. Electron-impact ionization mass spectrum of the peak at scan no. 253 (Fig. 8). This peak was recognized as having been generated by hexadecanol.

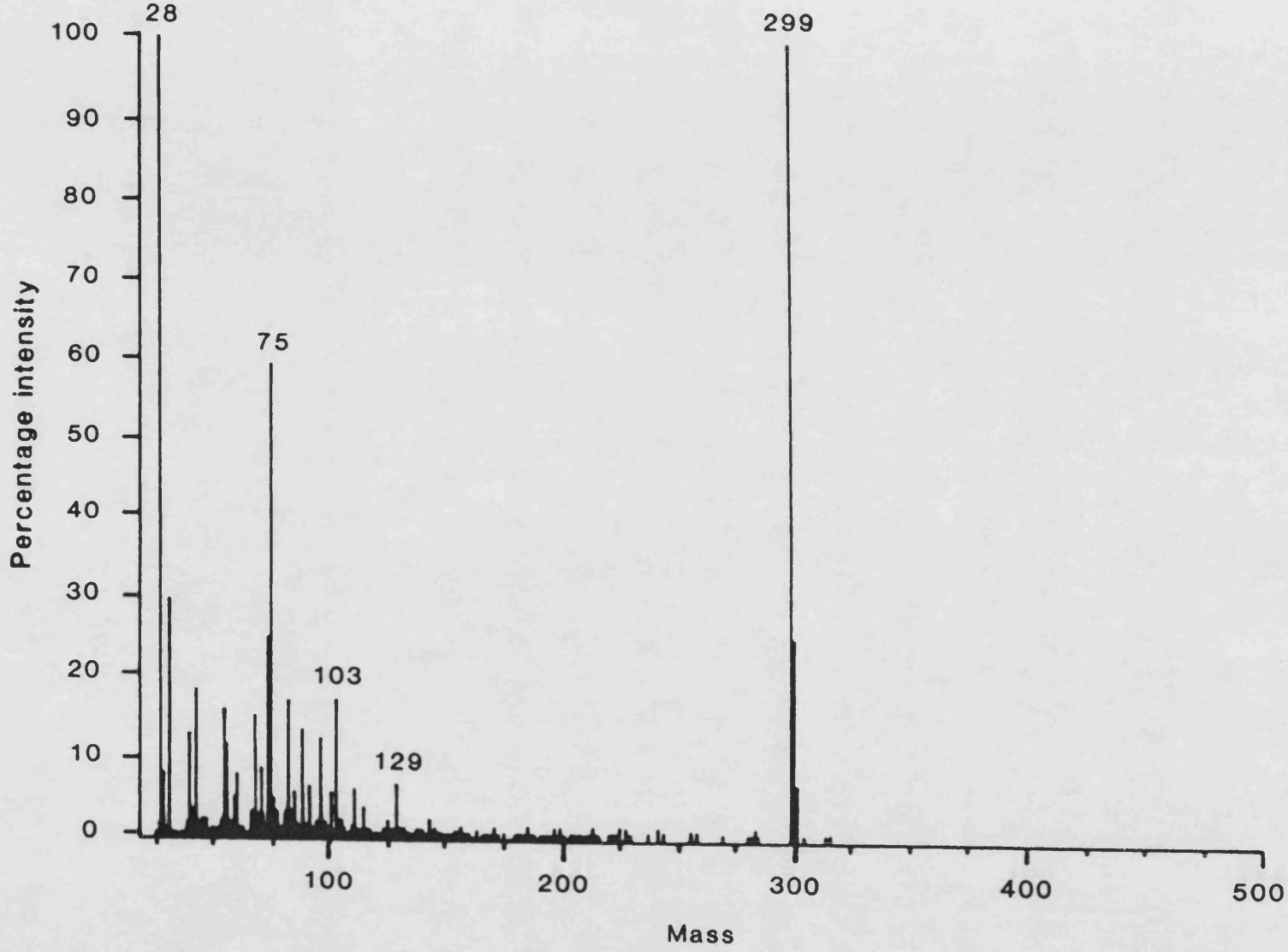
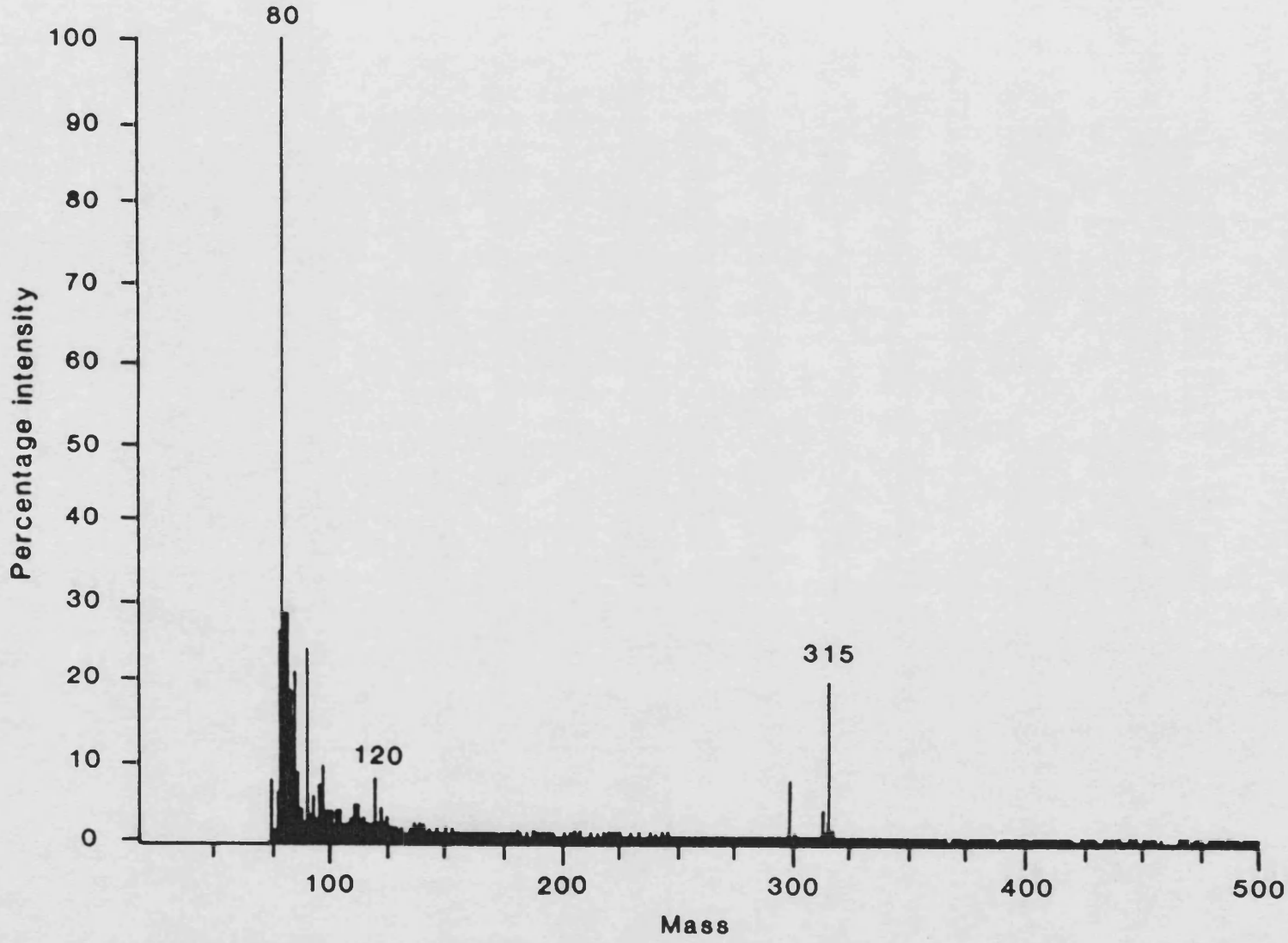

28
299
75
103
129
100
90
80
70
60
50
40
30
20
10
0
Percentage intensity
100
200
300
400
500
Mass

Figure 10. Chemical ionization mass spectrum of the hexadecanol peak at scan no. 253 (Fig. 8).


80
100
90
80
70
60
50
40
30
20
10
0
Percentage intensity
120
315
100
200
300
400
500
Mass

Survey of Long-Chain Alcohol Production by Yeasts

A survey was made of the ability of 14 strains of yeast to produce long-chain alcohols. Strains were selected partly on the basis of their known ability to metabolize exogenously provided lipids. All strains grew under aerobic conditions although five were unable to do so under self-induced anaerobic conditions (Table 4). While some of the strains produced saturated long-chain alcohols, none produced unsaturated long-chain alcohols. Only three yeasts, C. albicans, C. utilis NCYC 168 and P. fermentans, accumulated appreciable amounts of long-chain alcohols when grown either self-induced anaerobically or aerobically (Table 4). Candida maltosa, C. utilis NCYC 707 and Sacch. cerevisiae contained these alcohols when grown under self-induced anaerobic conditions, but little or no such alcohol after aerobic growth. In general, self-induced anaerobic conditions appeared to favour production of long-chain alcohols.

Six of the yeast strains with the ability to produce long-chain alcohols under self-induced anaerobic conditions were examined quantitatively for content of $C_{14:0}$, $C_{16:0}$ and $C_{18:0}$ alcohols (Table 5). The content of hexadecanol was greatest after self-induced anaerobic growth with all of the strains examined. With the one yeast grown under aerobic conditions, octadecanol was found in slight excess over hexadecanol, although the total long-chain alcohol content was approximately four-fold greater in organisms from self-induced anaerobically-grown cultures of this yeast. Production of all alcohols, especially hexadecanol, was greatest by C. albicans grown under self-induced anaerobic conditions. Only one

Table 4. Detection of long-chain alcohols in yeasts grown under self-induced anaerobic or aerobic conditions for 72 h. Each analysis was conducted two times.

Yeast strains	Detection of long-chain alcohols in organisms grown under: Self-induced anaerobic conditions			Aerobic conditions		
	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$
Candida albicans NCYC 1467	+	+	+	+	+	+
Candida bombicola NCYC 1449	-	-	-	-	-	-
Candida ingens NCYC 822	no growth			-	-	-
Candida maltosa CMCC 3152	+	+	+	-	-	-
Candida utilis NCYC 168	+	+	+	+	+	+
Candida utilis NCYC 707	+	+	+	-	-	-
Debaryomyces hansenii NCYC 9	-	-	-	-	-	-
Pichia fermentans NCYC 850	+	+	+	+	+	+
Rhodotorula glutinis NCYC 59	-	-	-	-	-	-
Rhodotorula glutinis CMCC 2272	no growth			-	-	-
Rhodotorula rubra NCYC 195	no growth			+/-	+/-	+/-
Rhodotorula sp. ATCC 20254	no growth			-	-	-
Saccharomyces cerevisiae Y185	+	+	+	+/-	+/-	+/-
Torulopsis petrophilum ATCC 20225	no growth			-	-	-

Table 5. Long-chain alcohol contents of selected yeast strains grown under self-induced anaerobic conditions (except where otherwise indicated) for 72 h. Values quoted are the means of three independent determinations ± SD.

Yeast strains	Long-chain alcohol content (μg (g dry wt organsims)$^{-1}$)		
	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$
Candida albicans NCYC 1467 (aerobic)	8.76 ± 0.80	18.08 ± 0.97	26.31 ± 2.03
Candida albicans NCYC 1467	20.59 ± 1.79	139.29 ± 10.24	34.47 ± 2.82
Candida maltosa CMCC 3152	1.76 ± 0.35	47.91 ± 13.84	5.62 ± 1.01
Candida utilis NCYC 168	3.30 ± 0.63	17.93 ± 3.47	3.92 ± 0.82
Candida utilis NCYC 707	2.13 ± 0.72	27.05 ± 9.26	4.42 ± 0.98
Pichia fermentans NCYC 850	3.25 ± 0.30	28.47 ± 7.88	4.38 ± 0.68
Saccharomyces cerevisiae Y185	0.89 ± 0.02	4.66 ± 0.48	1.35 ± 0.55

of the other five strains, C. maltosa, synthesized more than 25% of the total long-chain alcohol produced by C. albicans under these conditions.

Further studies on long-chain alcohol production were confined to C. albicans.

Growth and Production of Long-Chain Alcohols by Candida albicans Under Self-Induced Anaerobic Conditions

Candida albicans had a generation time of 1h 50 min, and reached the stationary phase of growth after 48 h incubation with a final growth yield of 3.5 mg dry wt ml^{-1} (Fig. 11). Only traces of long-chain alcohols were detected in organisms from exponentially growing cultures. However, after cultures entered the stationary phase of growth, the content of $C_{14:0}$, $C_{16:0}$ and $C_{18:0}$ alcohols increased rapidly until cultures were 168 h old after which alcohol contents remained constant. Rates of increase in long-chain alcohol content during the stationary phase of growth were greatest for hexadecanol (3.0 μg h^{-1}) and smallest for tetradecanol (1.2 μg h^{-1}). Octadecanol had a maximal rate of production of 1.7 μg h^{-1} (Fig. 11). Long-chain alcohols were not detected in the culture medium except after prolonged (three weeks) incubation, when the low concentrations present were probably due to limited lysis of organisms.

Effect of Glucose on Long-Chain Alcohol Production by Candida albicans

Due to the fact that the media used for self-induced anaerobic

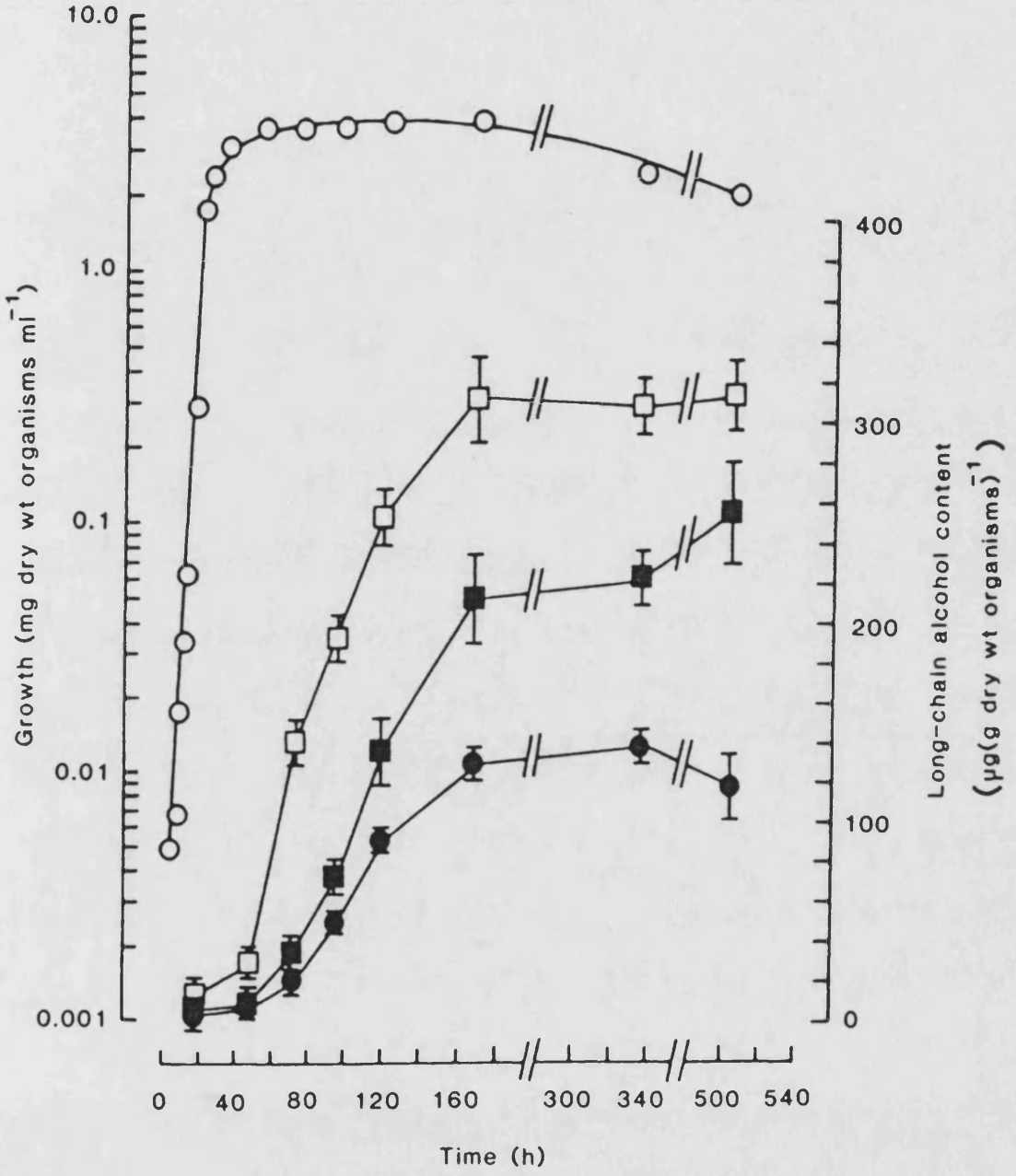


Figure 11. Time-course of self-induced anaerobic growth (O) of Candida albicans and of its contents of $C_{14:0}$ (●), $C_{16:0}$ (□) and $C_{18:0}$ (■) long-chain alcohols. Values plotted are the means of three independent determinations ± SD.

and aerobic growth differed in glucose concentration, a study was made of the effect of glucose concentration on production of long-chain alcohols by C. albicans. Organisms grown aerobically in media containing 200 g glucose and 4.0 g yeast extract l^{-1} had a generation time of 1 h 10 min, and reached the stationary phase of growth after 48 h incubation with a final growth yield of 7.5 mg dry wt ml^{-1} (Fig. 12). Like organisms grown under self-induced anaerobic conditions, the three major saturated alcohols were produced only after cessation of exponential growth. While the content of $C_{14:0}$ alcohols was always lower than those of $C_{16:0}$ and $C_{18:0}$ alcohols, the pattern of alcohol production differed from that encountered during self-induced anaerobic growth in that the contents of $C_{16:0}$ and $C_{18:0}$ alcohols were very similar (Fig. 12). As the concentration of glucose in media used for aerobic growth of organisms was raised from 1.0 to 30.0% (w/v), the contents of all three major classes of long-chain alcohol increased in organisms from 168 h cultures to a maximum total content of approximately 900 µg (g dry wt organisms)$^{-1}$ (Fig. 13). At all glucose concentrations, under aerobic growth conditions, the $C_{18:0}$ alcohol content of organisms was in slight excess over $C_{16:0}$ alcohol and, as the glucose concentrations increased, so did the relative proportions of $C_{14:0}$ alcohol. However, in organisms grown under self-induced anaerobic conditions for 168 h, peak contents of each of the three long-chain alcohol classes were greatest for organisms grown in media containing 10.0% (w/v) glucose amounting to a total of approximately 980 µg (g dry wt organisms)$^{-1}$ (Fig. 14). At all glucose concentrations, except 1.0% (w/v), $C_{16:0}$ alcohol

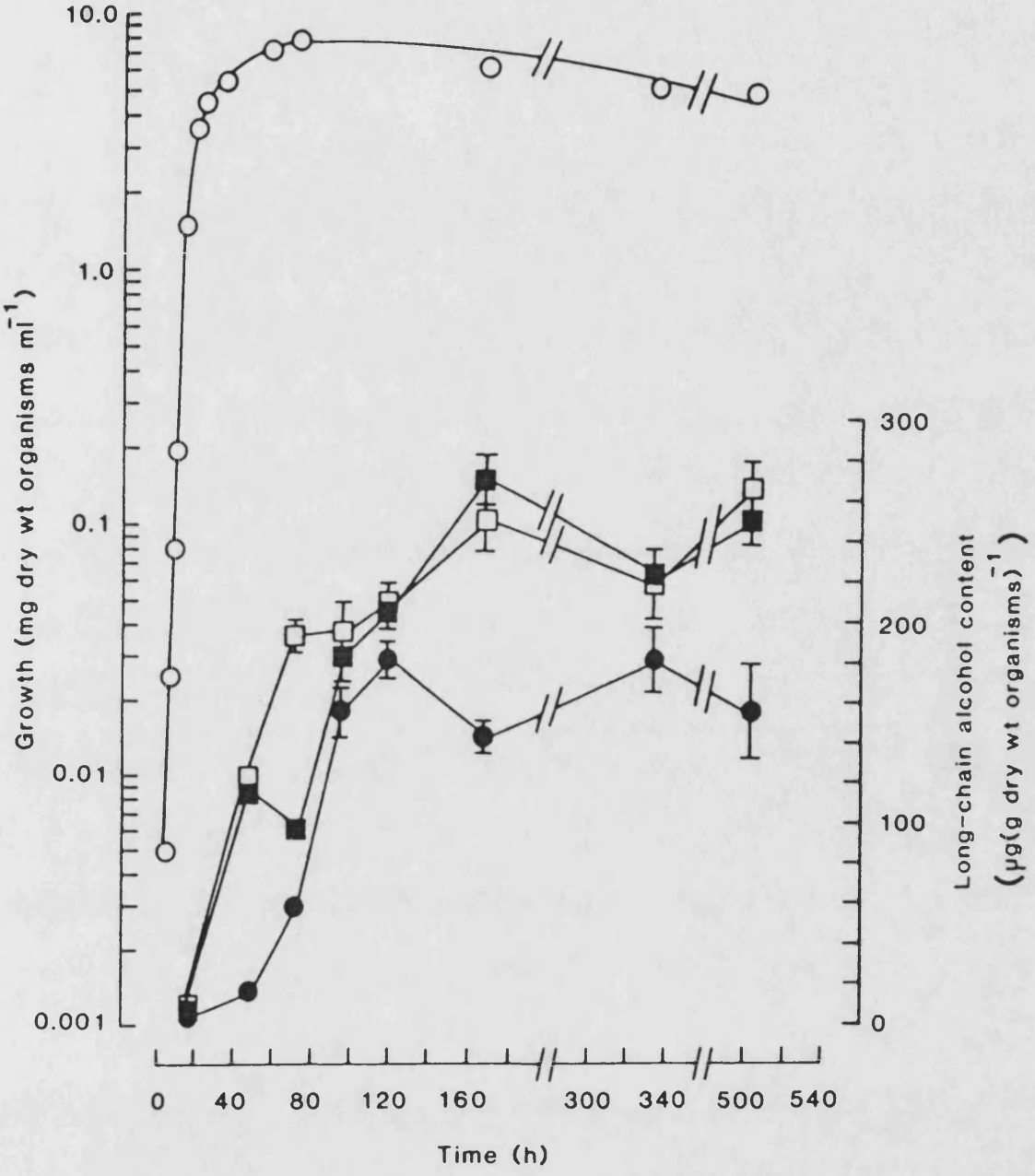


Figure 12. Time-course of aerobic growth (O) of Candida albicans in media containing 200 g glucose and 4.0 g yeast extract l^{-1}, and of its contents of $C_{14:0}$ (●), $C_{16:0}$ (□) and $C_{18:0}$ (■) long-chain alcohols. Values plotted are the means of three independent determinations ± SD.

Figure 13. Effect of glucose concentration on long-chain alcohol contents of *Candida albicans* grown under aerobic conditions for 168 h. The number at the base of each bar indicates saturated alcohol chain-length. Each bar represents the mean of three independent determinations ± SD.

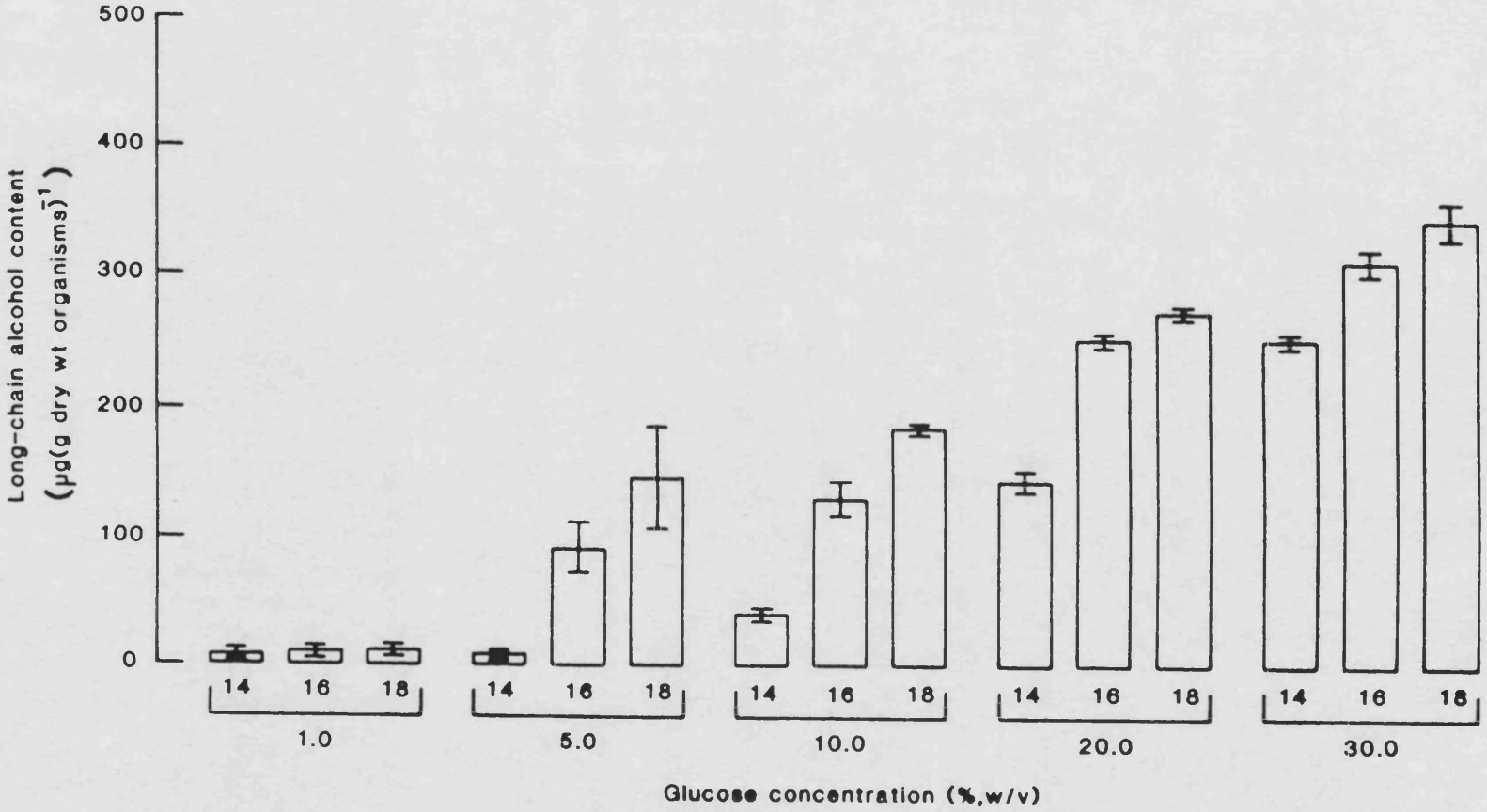
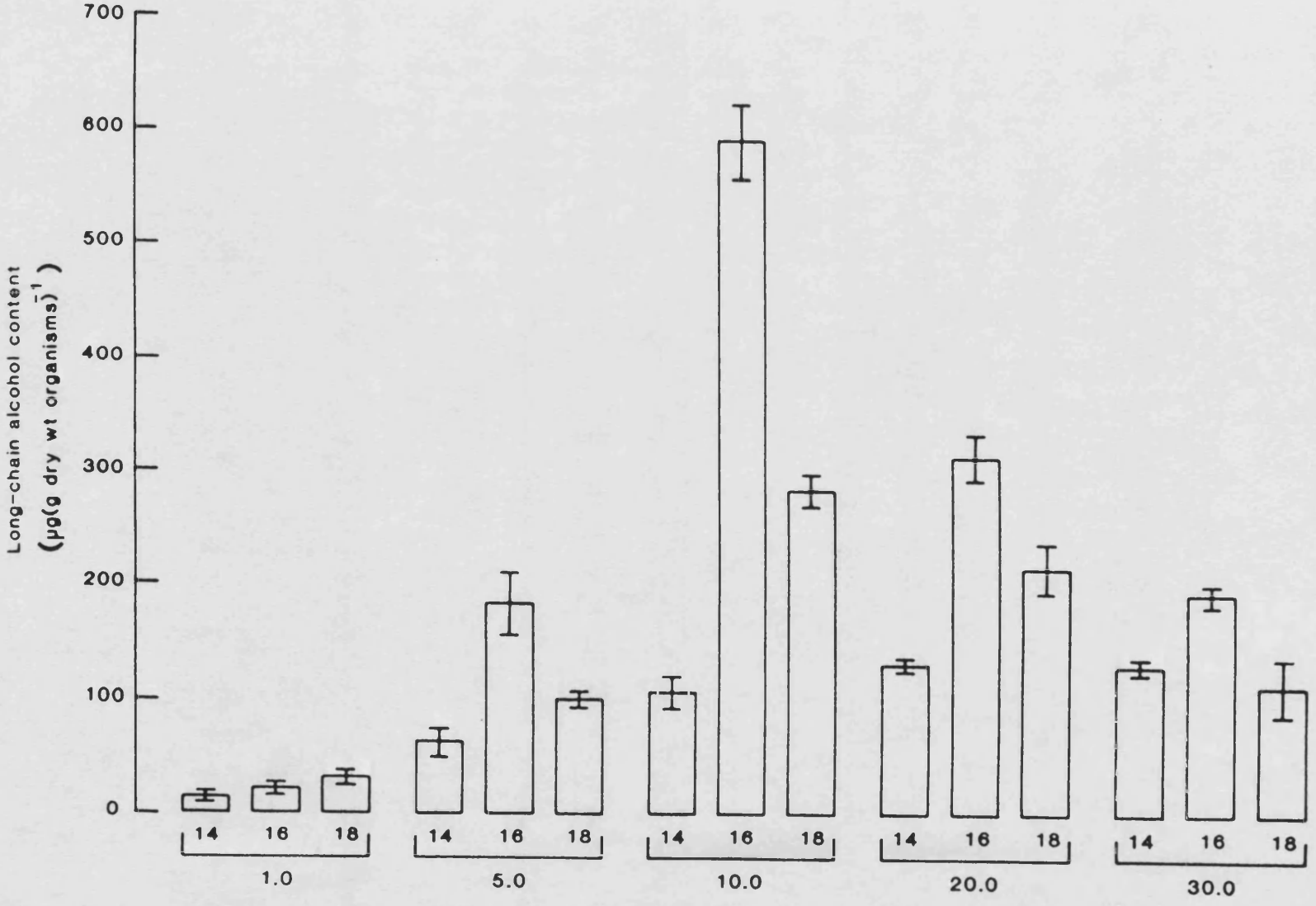

Long-chain alcohol content
(μg(g dry wt organisms)⁻¹)
500
400
300
200
100
0
14
16
18
1.0
5.0
10.0
20.0
30.0
Glucose concentration (%,w/v)

Figure 14. Effect of glucose concentration on long-chain alcohol contents of *Candida albicans* grown under self-induced anaerobic conditions for 168 h. The number at the base of each bar indicates saturated alcohol chain-length. Each bar represents the mean of three independent determinations ± SD.


Long-chain alcohol content
(μg(g dry wt organisms)⁻¹)
700
600
500
400
300
200
100
0
14
16
18
1.0
5.0
10.0
20.0
30.0
Glucose concentration (%,w/v)

predominated. Each culture harvested after 168 h incubation had been in the stationary phase of growth for approximately the same length of time.

Effect of the Nature of the Carbon Source on Long-Chain Alcohol Production by Candida albicans

Substituting glucose (10%, w/v) by the same concentration of galactose greatly decreased the total long-chain alcohol content in organisms from 352 μg to 133 μg (g dry wt organisms)$^{-1}$, when grown aerobically for 168 h (Table 6). Proportionally, the effect was greatest for $C_{14:0}$ alcohols and smallest for $C_{18:0}$ alcohols. Replacing glucose by glycerol (10%, w/v) rendered C. albicans incapable of producing quantifiable amounts of long-chain alcohol (Table 6). Each culture harvested after 168 h incubation had been in the stationary phase of growth for approximately the same length of time.

Fatty-Acyl Composition of Total Lipids and Lipid Classes in Candida albicans

Organisms grown for 168 h, under self-induced anaerobic conditions or aerobic conditions in media containing 4.0 g yeast extract and 50 or 200 g glucose l^{-1}, were analysed for their fatty-acyl composition (Tables 7-9). In general, the degree of unsaturation (Δ mol^{-1}) for all lipid classes, under these growth conditions, was lowest in organisms grown under self-induced anaerobic conditions and highest for those grown aerobically in media containing 5% (w/v) glucose (Tables 7-9). The major

Table 6. Effect of carbon source on the long-chain alcohol content of Candida albicans. Organisms were grown aerobically for 168 h. Values quoted are the means of three independent determinations ± SD. nd indicates that the alcohol was not detected, tr that a trace was detected.

Carbon source (10%, w/v)	Long-chain alcohol content (μg (g dry wt organisms)$^{-1}$)		
	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$
Glucose	37.1 ± 5.3	130.0 ± 11.2	184.9 ± 3.9
Galactose	3.7 ± 0.3	33.7 ± 2.7	95.3 ± 11.2
Glycerol	nd	tr	tr

Table 7. Fatty-acyl composition of total lipids and lipid classes from Candida albicans grown under self-induced anaerobic conditions for 168 h. Values quoted are the means of three independent determinations ± SD. Δmol^{-1} values were calculated as described by Kates and Hagen (1964).

Fatty-acyl chain	Contents of fatty-acyl residues (percentage of total) in:				
	Total lipids	Phospholipids	Free fatty acids	Triacyl-glycerols	Sterol/wax esters
12:0	11.3 ± 1.3	1.5 ± 0.2	7.3 ± 2.0	11.6 ± 1.1	14.5 ± 1.7
14:0	18.4 ± 1.2	4.3 ± 0.5	7.4 ± 0.9	20.6 ± 1.9	13.3 ± 0.5
16:0	25.9 ± 1.3	25.4 ± 1.0	24.3 ± 0.7	22.8 ± 0.8	23.5 ± 1.6
16:1	6.7 ± 0.6	6.3 ± 0.6	7.4 ± 3.9	5.3 ± 0.5	6.3 ± 1.5
18:0	15.2 ± 1.8	18.3 ± 0.3	27.1 ± 6.0	21.3 ± 1.6	22.2 ± 0.8
18:1	7.8 ± 0.1	16.2 ± 1.8	10.3 ± 1.0	7.1 ± 0.6	8.7 ± 1.1
18:2	13.3 ± 0.9	25.2 ± 1.2	14.2 ± 0.9	9.9 ± 0.3	10.2 ± 1.2
18:3	1.7 ± 0.2	2.8 ± 0.1	2.1 ± 0.5	1.3 ± 0.1	1.4 ± 0.3
Δmol^{-1}	0.46	0.81	0.52	0.36	0.40

Table 8. Fatty-acyl composition of total lipids and lipid classes from Candida albicans grown under aerobic conditions for 168 h in media containing 200 g glucose and 4.0 g yeast extract l^{-1}. Values quoted are the means of three independent determinations ±SD. Δmol^{-1} values were calculated as described by Kates and Hagen (1964). nd indicates that the fatty-acyl residue was not detected.

Fatty-acyl chain	Contents of fatty-acyl residues (percentage of total) in:				
	Total lipids	Phospholipids	Free fatty acids	Triacyl-glycerols	Sterol/wax esters
12:0	nd	nd	nd	nd	nd
14:0	4.0 ± 0.0	0.5 ± 0.0	2.6 ± 0.3	4.9 ± 0.1	3.0 ± 0.4
16:0	16.2 ± 0.5	25.7 ± 0.3	21.9 ± 0.1	14.1 ± 0.5	18.0 ± 2.2
16:1	20.7 ± 0.2	15.3 ± 0.3	13.1 ± 1.2	23.9 ± 0.1	14.2 ± 0.4
18:0	4.9 ± 0.1	5.7 ± 0.2	10.3 ± 0.1	4.2 ± 0.2	6.3 ± 0.3
18:1	40.9 ± 1.1	39.2 ± 0.9	42.3 ± 1.3	40.7 ± 1.4	41.5 ± 3.5
18:2	12.4 ± 0.6	12.7 ± 1.1	9.9 ± 0.2	11.4 ± 0.9	15.3 ± 0.8
18:3	1.1 ± 0.1	1.0 ± 0.1	nd	0.9 ± 0.1	1.7 ± 0.0
Δmol^{-1}	0.89	0.83	0.75	0.90	0.91

Table 9. Fatty-acyl composition of total lipids and lipid classes from Candida albicans grown under aerobic conditions for 168 h in media containing 50 g glucose and 4.0 g yeast extract l^{-1}. Values quoted are the means of three independent determinations ± SD. Δmol^{-1} values were calculated as described by Kates and Hagen (1964). nd indicates that the fatty-acyl residue was not detected.

Fatty-acyl chain	Contents of fatty-acyl residues (percentage of total) in:				
	Total lipids	Phospholipids	Free fatty acids	Triacyl-glycerols	Sterol/wax esters
12:0	nd	nd	nd	nd	nd
14:0	3.6 ± 0.7	2.5 ± 0.3	3.1 ± 0.4	5.6 ± 1.4	4.3 ± 0.3
16:0	21.6 ± 0.8	29.9 ± 0.5	20.7 ± 1.6	15.3 ± 0.6	17.5 ± 0.1
16:1	13.1 ± 0.7	13.1 ± 0.0	15.6 ± 7.1	17.6 ± 1.2	7.3 ± 0.2
18:0	7.2 ± 0.3	7.1 ± 0.1	13.7 ± 3.6	5.4 ± 0.1	7.5 ± 0.1
18:1	24.1 ± 1.0	21.3 ± 0.4	21.2 ± 2.3	25.0 ± 0.1	25.3 ± 0.1
18:2	26.5 ± 0.9	23.4 ± 0.4	22.8 ± 0.1	27.4 ± 3.5	32.7 ± 0.2
18:3	3.9 ± 0.0	2.8 ± 0.1	2.9 ± 0.2	3.8 ± 0.3	5.2 ± 0.4
Δmol^{-1}	1.02	0.90	0.91	1.09	1.14

fatty-acyl residues in all lipids analysed were C_{16} and C_{18} (combined saturated and unsaturated residues). Under aerobic conditions (Tables 8 and 9), the relative proportions of C_{18} fatty-acyl residues in all lipid classes were higher than those found under self-induced anaerobic conditions (Table 7). More notable was the relatively high proportion of shorter chain fatty-acyl residues ($C_{12:0}$ and $C_{14:0}$) in triacylglycerol and sterol/wax ester fractions of self-induced anaerobically grown organisms and, the higher proportion of unsaturated fatty-acyl residues in the triacylglycerol and sterol/wax ester fractions of aerobically grown organisms. Fatty-acyl residues with chain-lengths shorter than C_{14} were not detected in the aerobically grown cultures examined and $C_{14:0}$ residues, although present in detectable proportions, were also reduced.

Long-Chain Alcohol Production in Self-Induced Anaerobic Cultures of *Candida albicans* Supplemented with Long-Chain Fatty Acids

Organisms grown for 168 h in media supplemented with 10 mg $C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{18:0}$ or $C_{18:1}$ fatty acid l^{-1} did not show any deviation in their long-chain alcohol contents from those grown in unsupplemented media. However, when organisms were supplemented with the odd chain-length fatty acids $C_{13:0}$, $C_{15:0}$, $C_{17:0}$ and $C_{19:0}$, there was an appearance of odd chain-length alcohols in their lipid extracts (Table 10). These odd chain-length alcohols, which were never found under unsupplemented conditions, co-chromatographed with authentic alcohol standards and their identities were confirmed by GLC-mass spectrometry. The contents of

Table 10. Long-chain alcohol composition of Candida albicans grown in media supplemented with long-chain fatty acids. Organisms were grown under self-induced anaerobic conditions for 168 h in media containing 20% (w/v) glucose and 10 mg l^{-1} of the long-chain fatty acid indicated. Values quoted are the means of three independent determinations ± SD. nd indicates that the alcohol was not detected, tr that a trace was detected.

Fatty acid supplement	Long-chain alcohol content (μg (g dry wt organisms)$^{-1}$)						
	$C_{13:0}$	$C_{14:0}$	$C_{15:0}$	$C_{16:0}$	$C_{17:0}$	$C_{18:0}$	$C_{19:0}$
None	nd	129.9 ± 4.1	nd	312.4 ± 19.2	nd	213.1 ± 22.0	nd
$C_{13:0}$	26.3 ± 0.2	100.2 ± 5.0	22.4 ± 0.3	296.9 ± 6.1	tr	146.2 ± 5.1	nd
$C_{15:0}$	tr	115.4 ± 1.6	28.5 ± 1.0	319.4 ± 14.5	tr	206.8 ± 5.8	nd
$C_{17:0}$	nd	133.1 ± 5.7	tr	307.2 ± 6.0	85.5 ± 4.5	202.4 ± 7.6	tr
$C_{19:0}$	nd	132.7 ± 9.3	tr	323.1 ± 3.2	9.6 ± 1.0	200.0 ± 7.6	5.3 ± 0.3

odd chain-length alcohols in organisms were very low compared with the contents of the three major even chain-length alcohols. Maximum conversion of odd chain-length fatty acid to odd chain-length alcohol was achieved using a heptadecanoic acid supplement, producing 85.5 μg $C_{17:0}$ alcohol (g dry wt organisms)$^{-1}$. There was also some evidence for chain elongation and β-oxidation when shorter and longer odd chain-length fatty acid substrates were used (Table 10). When a $C_{13:0}$ acid supplement was used, there was approximately the same amount of $C_{15:0}$ alcohol produced as $C_{13:0}$ alcohol, and when $C_{19:0}$ acid was used, there was slightly more $C_{17:0}$ alcohol produced than $C_{19:0}$ alcohol.

Supplementing cultures every 24 h, after an initial 24 h incubation, with 2 mg heptadecanoic acid l^{-1} to a final concentration of 10 mg l^{-1} increased the amount of $C_{17:0}$ alcohol produced from 85.5 μg to 153.9 μg (g dry wt organisms)$^{-1}$ (Tables 10 and 11). Moreover, the effect of glucose concentration on synthesis of $C_{17:0}$ alcohols (Table 11) by organisms grown under self-induced anaerobic conditions for 168 h, was similar to the effect on production of the three major even chain-length alcohols (Fig. 14). There was least production of heptadecanol in cultures containing 1.0% (w/v) glucose, rising to a maximum in cultures containing 10.0% (w/v) glucose and then a decrease in production with glucose concentrations above 10.0% (w/v). Production of $C_{17:0}$ alcohols under these conditions also led to a decrease in contents of $C_{16:0}$ and $C_{18:0}$ alcohols, while the $C_{14:0}$ alcohol content remained unaltered (Table 11 and Fig. 14).

Table 11. Effect of glucose concentration on long-chain alcohol content of Candida albicans grown under self-induced anaerobic conditions with repeated long-chain fatty acid supplementation. Cultures were grown for 168 h, and supplemented with 2 mg $(24\ h)^{-1}$ heptadecanoic acid to a final concentration of 10 mg l^{-1}. Values quoted are the means of three independent determinations ± SD.

Glucose concentration (%, w/v)	Long-chain alcohol content (μg (g dry wt organsims)$^{-1}$)			
	$C_{14:0}$	$C_{16:0}$	$C_{17:0}$	$C_{18:0}$
1.0	6.8 ± 0.9	24.0 ± 1.8	31.2 ± 6.3	17.0 ± 0.6
5.0	56.0 ± 7.2	204.7 ± 20.8	38.6 ± 3.1	134.7 ± 16.7
10.0	151.9 ± 6.4	427.0 ± 4.6	183.8 ± 8.6	228.5 ± 1.6
20.0	144.1 ± 7.3	221.3 ± 10.7	153.9 ± 7.5	133.2 ± 8.1
30.0	124.9 ± 19.9	114.9 ± 10.4	62.7 ± 8.1	46.6 ± 9.7

Production of Hexadecanol by Cell-Free Extracts of Candida albicans

Both microsomal and soluble protein fractions, from C. albicans grown under self-induced anaerobic conditions for 72 h, reduced palmitoyl-CoA to the corresponding primary alcohol, namely hexadecanol (Table 12). The mean rate of production of hexadecanol using microsomal preparations was 0.14 nmol h^{-1} (mg protein)$^{-1}$ irrespective of whether NADH or NADPH was used as the reductant. A faster rate of 0.70 nmol h^{-1} (mg protein)$^{-1}$ was obtained using soluble protein preparations. Again, the same rate was obtained using either NADH or NADPH.

Effect of pCMB on Contents of Free and Esterified Long-Chain Alcohols in Candida albicans

Organisms from cultures grown under self-induced anaerobic conditions for 120 h showed lipase activities with pH optima around 9.0 using either olive oil or tributyrin as the substrate. When organisms were pre-incubated with pCMB (final concentration 0.5 mM) there was a significant decrease (approximately 60%) in lipase activity when tributyrin was used as the substrate (Table 13). The lipase inhibitor pCMB (Schousboe, 1976) was added as a methanol solution. When organisms were pre-incubated with methanol alone, there was no change in lipase activities (Table 13).

Supplementing cultures with pCMB just prior to harvesting, and including this compound during the lipid extraction procedure up to the stage at which filtered chloroform/methanol extracts were pooled, appreciably decreased the apparent content of the three major even-chain alcohols (Table 14). The possibility that a

Table 12. Production of hexadecanol by cell-free extracts of *Candida albicans*. Organisms were grown under self-induced anaerobic conditions for 72 h. Values quoted are the means of three independent determinations ± SD.

Subcellular fraction	Reductant	Hexadecanol production (nmol h^{-1} (mg protein)$^{-1}$)
Microsomal	NADH	0.13 ± 0.05
Microsomal	NADPH	0.14 ± 0.04
Soluble protein	NADH	0.68 ± 0.08
Soluble protein	NADPH	0.70 ± 0.06

Table 13. Lipase activities (pH 9.0) of Candida albicans (whole organisms). Organisms were grown under self-induced anaerobic conditions for 120 h. One lipase unit (U) corresponds to 1 μmol fatty acid released min^{-1}. Values quoted are the means of three independent determinations ± SD.

Substrate	Treatment	Lipase activity (U (g dry wt organisms)$^{-1}$
Olive oil	None	230.9 ± 22.9
Tributyrin	None	187.5 ± 6.1
Tributyrin	pCMB in MeOH	77.9 ± 10.4
Tributyrin	MeOH	191.2 ± 4.2

Table 14. Effect of pCMB on contents of free and esterified long-chain alcohols in Candida albicans. Organisms were grown under self-induced anaerobic conditions for 120 h. pCMB was included before harvesting and during the extraction procedure as indicated in the text. Values quoted are the means of three independent determinations ± SD.

Inhibitor	Fraction	Long-chain alcohol content (μg (g dry wt organisms)$^{-1}$)		
		$C_{14:0}$	$C_{16:0}$	$C_{18:0}$
None	Free alcohol	90.6 ± 5.7	252.9 ± 10.5	135.7 ± 14.9
	Wax ester	8.2 ± 0.6	7.5 ± 0.1	6.5 ± 0.2
pCMB	Free alcohol	55.8 ± 3.0	126.0 ± 16.8	61.1 ± 3.9
	Wax ester	19.5 ± 0.5	22.9 ± 0.7	12.1 ± 0.6

proportion of the alcohols was esterified was therefore examined using unsupplemented extractions and extractions containing pCMB. Only a small proportion was found to be esterified in this fraction (Table 14). The relative proportions of alcohol chain-lengths in the wax-ester fractions were also different from those found with the free long-chain alcohols.

DISCUSSION

The work reported in this thesis involved two aspects of fatty-acid metabolism. For this reason the discussion has been separated into two major sections.

OLEIC ACID INCORPORATION INTO ANAEROBICALLY GROWN *SACCHAROMYCES CEREVISIAE* Y185

The Zymology Laboratory in the University of Bath has for some years been interested in composition-function relationships in the plasma membrane of *Sacch. cerevisiae*. The technique that has been extensively used to enrich plasma-membrane lipids exploits the anaerobically-induced requirement that *Sacch. cerevisiae* has for an unsaturated fatty acid and a sterol. However, there have been no previous attempts to quantify the incorporation of fatty acids into this organism or indeed to establish the degree to which these fatty acids are associated with other subcellular organelles. Furthermore, it is unclear as to how exogenous fatty acids are incorporated into the plasma membranes of anaerobically grown *Sacch. cerevisiae*.

In this thesis, initial studies on the growth characteristics of *Sacch. cerevisiae* Y185 under strict anaerobic conditions in media supplemented with oleic acid and ergosterol were in agreement with previous reports using this yeast (Calderbank, 1984; Calderbank *et* al., 1984; 1985). These workers were only concerned with enriching plasma-membrane phospholipids with unsaturated fatty-acyl residues as far as studies on accumulation of amino

acids were concerned. Therefore they were only interested in relative proportions of plasma-membrane enrichment rather than absolute amounts. In many of the anaerobic studies using Sacch. cerevisiae (Calderbank, 1984; Calderbank et al., 1984; 1985; Keenan et al., 1982; Thomas and Rose, 1979; Thomas et al., 1978; Watson and Rose, 1980) cultures were supplemented with 30 mg unsaturated fatty acid l^{-1} without any knowledge of the quantity utilized by the organisms. The present work, using Sacch. cerevisiae Y185 grown anaerobically in the presence of [1-^{14}C]oleic acid, has revealed that organisms harvested from mid exponential-phase cultures (an arbitrary point that has been taken for studying the lipid biochemistry and physiology of yeast plasma membranes) only incorporated approximately 50% of the fatty acid supplement. Removal of the cell walls brought about a decrease in oleic acid associated with organisms. This may have been due to removal of oleoyl residues that had been incorporated into neutral lipids and glycerophospholipids of the cell walls (Nurminen and Suomalainen, 1969; Suomalainen and Nurminen, 1970). Alternatively, this loss could have been due to removal of free oleic acid that had adsorbed to the cell walls (Kohlwein and Paltauf, 1983). The latter possibility is the more likely explanation since the lipid found to be present in yeast cell walls is quite often due to preparations being contaminated with plasma-membrane fragments.

Turning to the subcellular location of residues of supplemented unsaturated fatty acids, studies using [1-^{14}C]oleic acid in the growth medium showed that this supplement was predominantly incorporated into low-density vesicles and plasma membranes. The

plasma membranes, identified by transmission electron microscopy and plasma-membrane ATPase marker enzyme studies, separated into three fractions on a discontinuous sucrose-density gradient. Closer examination of these plasma membranes revealed different degrees of vesicular association. In the majority of cases, plasma membranes are usually isolated either as a single band (Wehrli *et al.*, 1975) or as a pellet (Aldermann and Höfer, 1984; Marriott, 1975) after density centrifugation. However, Henschke *et al.* (1983), using aerobically grown *Sacch. cerevisiae* NCYC 366, found a turbid band which appeared at an intermediate density on a discontinuous sucrose-density gradient between the plasma-membrane pellet and low-density vesicles. Transmission electron microscopy showed that this band was composed of membranes with associated intracellular vesicles. These membranes were identified as being plasma membranes by isolating associations from ^{125}I-labelled spheroplasts. Furthermore, Henschke and his co-workers found that incubating crude plasma-membranes with isolated low-density vesicles led to formation of two visible intermediate-density bands when the incubation mixture was fractionated on a gradient similar to that used for separating membranes and low-density vesicles from spheroplast lysates. They noted that, as both density bands were generated, there was a linear decrease in the size of the plasma-membrane pellet as judged by protein content. The size of vesicles associated with plasma membranes isolated from anaerobically grown cultures of *Sacch. cerevisiae* Y185 are smaller than those reported by Henschke and his co-workers, but despite this, it is quite probable that the vesicular associations with the plasma membranes

of Sacch. cerevisiae Y185 are responsible for their separation into three fractions with slightly altered densities.

The low-density vesicle fraction isolated from Sacch. cerevisiae Y185 corresponded to the lowest density in the discontinuous sucrose-density gradient. Several workers have isolated vacuoles (Indge, 1968; Schwencke, 1977) and vesicles (Clausen et al., 1974; Schaffner and Matile, 1981) from Sacch. cerevisiae. However, very few workers have commented on the possibility that their preparations contain both types of subcellular organelle due to the similar procedures used to isolate them. Hossack et al. (1973) obtained a vesicle fraction which remained at the top of a discontinuous sucrose-density gradient at a density of 1.080 g ml^{-1} and this was believed to contain vacuoles as well as low-density vesicles (Cartledge and Rose, 1973). Vacuoles were not found in the vesicle fractions isolated in this present study. The low-density vesicles had a density corresponding to 1.035 g ml^{-1} and their purity was verified by transmission electron microscopy. The size of these low-density vesicles (0.25–0.50 µm diam.) however, is not consistent with those that have been reported for other strains of Sacch. cerevisiae. Cartledge et al. (1977) isolated two populations of vesicles from aerobically grown Sacch. cerevisiae NCYC 366 which differed in diameter. Henschke et al. (1983), using the same yeast strain, confirmed these findings and suggested that they were identical with those described by Schaffner and Matile (1981), mainly on the basis of their similarity in size (approximately 0.50–0.70 µm diam.)

While this difference in size of low-density vesicles could be due to strain variability, it must be emphasized that the cultural conditions used by the above mentioned workers were not anaerobic, nor were the cultures supplemented with oleic acid. Another important variable to be considered, which may affect the size of low-density vesicles, is the stage of growth at which organisms are harvested. Low-density vesicles have been reported to occur in both stationary-phase cultures of Sacch. cerevisiae (Matile et al., 1969) and in dividing cells, localized largely in the region of the developing bud (Sentandreu and Northcote, 1969) where they increase in diameter from around 0.1 to about 1.0 µm. I therefore feel that it is not unreasonable to suggest that the low-density vesicles found in anaerobically grown Sacch. cerevisiae Y185 probably have similar metabolic functions to those found in other yeasts.

A close association between vesicles and plasma membranes at the neck of budding Sacch. cerevisiae (Sentandreu and Northcote, 1969; Wiemken et al., 1970) is recognized as a vectorially important process in envelope growth in this and other eukaryotic micro-organisms (Rosenberger, 1979). This has prompted speculation on the way in which the contents of small vesicles contribute to growth of the plasma membrane and cell wall. While yeast vesicles are known to contain enzymes which might be involved in breakdown of cell-wall components (Cartledge et al., 1977; Cortat et al., 1972; Matile et al., 1971), and possibly in chitin synthesis in the bud scar (Cabib et al., 1982), they are known also to contain lipids (Cartledge et al., 1977; Clausen et al., 1974; Hossack et al., 1977; Schaffner and Matile, 1981). Incorporation of

[1-^{14}C]oleic acid into low-density vesicles and plasma membranes of Sacch. cerevisiae Y185 along with identification of plasma membrane/vesicle associations constitute the first evidence for involvement of low-density vesicles in exchange of lipids at the plasma membranes of yeasts. This possibility has in the past been proposed by several workers (Cartledge et al., 1977; Hossack et al., 1973; 1977; Schaffner and Matile, 1981). Further evidence for exchange of lipids between low-density vesicles and plasma membranes of anaerobically grown Sacch. cerevisiae Y185 was provided by shorter-term pulse-label experiments. The increase in relative [1-^{14}C]oleic acid activity in low-density vesicles and a corresponding decrease in the plasma membranes may suggest that the oleic acid supplement is initially taken up into vesicles before being incorporated into plasma-membrane phospholipids. Admittedly, one needs to examine this proposition in further detail, possibly involving even shorter-term pulse-label studies and pulse-chase experiments, before any firm conclusions can be made. However, the major obstacle to overcome in all of these studies with yeasts lies with the time taken to produce spheroplasts and fractionate organisms. The possibility of continued lipid metabolism during the formation of spheroplasts cannot be ruled out due to previous reports on fatty acid uptake and turnover of phospholipid fatty-acyl chains. Kohlwein and Paltauf (1983) studying uptake of fatty acids by yeasts found that, during the first 30 sec, 35% of the fatty acids taken up were incorporated into phospholipids. This rapid incorporation of fatty acids into cellular lipids has also been demonstrated in cultured neuroblastoma cells (Chakravarthy

et al., 1986). These latter workers showed that incorporation of essential fatty acids, with esterification primarily into plasma-membrane phospholipids, occurred with incubation times as short as 2 min.

Having established the location of incorporated oleic acid in anaerobically grown Sacch. cerevisiae Y185, the next question to be answered was in what form is it found? It was very interesting to find a large difference in the distribution of [1-^{14}C]oleoyl residues in lipids from whole cells and spheroplasts. This bears out the difficulties mentioned above that uncontrolled lipid metabolism continues throughout spheroplast formation. The most notable change in distribution was the transfer of free oleic acid to the triacylglycerol and sterol-ester fractions when organisms were converted to spheroplasts. Many authors have suggested that triacylglycerols might serve to regulate the types of fatty-acyl residues found in membrane phospholipids (Haley and Jack, 1977; Holub and Lands, 1975; Watson and Rose, 1980) and Taylor and Parks (1978, 1979) have shown that triacylglycerols and sterol esters accumulate in late exponential- and stationary-phase cultures of Sacch. cerevisiae which serve as a pool for membrane synthesis. I therefore propose that, in this study, transfer of oleic acid to triacylglycerol and sterol-ester fractions was due to an induced stationary phase in organisms, brought on by the conditions used for spheroplast formation. This transformation would have also eliminated any inherent toxicity of the free oleic acid (Boulton and Ratledge, 1984; Fukui and Tanaka, 1981).

From my results it is evident that incorporated oleic acid is present in low-density vesicles mainly in the form of triacylglycerols. This inferred high proportion of triacylglycerols in the low-density vesicles while being consistent with some reports on Sacch. cerevisiae (Clausen et al., 1974; Hossack et al., 1977) contradicts others (Cartledge et al., 1977; Schaffner and Matile, 1981). Discrepancies in analytical data may not only be due to the use of different strains of Sacch. cerevisiae and analytical methods used, but also to changes induced in the composition of low-density vesicles by environmental factors. Hossack et al. (1977) found that the vesicle population of anaerobically-grown Sacch. cerevisiae NCYC 366 supplemented with ergosterol and oleic acid, as compared with aerobically-grown organisms, had a much higher proportion of triacylglycerols and considerably less sterol ester. The low relative [1-^{14}C]activity of the sterol-ester fraction in the low-density vesicles isolated from Sacch. cerevisiae Y185 may also infer a low sterol-ester content. This would be consistent with the belief that exogenously provided sterol is incorporated directly into the plasma membrane under these conditions (Hossack et al., 1977).

Lipid analysis of isolated plasma membranes has shed more light on the involvement of low-density vesicles in plasma-membrane biosynthesis. In addition to triacylglycerols and sterol esters associated with the plasma membranes, there was also a high proportion of [1-^{14}C]oleoyl residues associated with the triacylglycerol fraction. This was almost certainly due to vesicular association. Furthermore, the distribution of [1-^{14}C]

oleoyl reisudes in the lipids of combined low-density vesicles and isolated plasma membranes resembled that found in unlysed spheroplasts. These results strongly support the observation made by Hossack et al. (1977). These workers revealed that the lipid content of low-density vesicles isolated from Sacch. cerevisiae NCYC 366 was lower than the cellular content of neutral lipids. They suggested that this was probably due to the fusion of small vesicles with the plasma membrane during membrane growth, which in turn would prevent them from being isolated as low-density vesicles.

The absence of diacylglycerols from low-density vesicles isolated from Sacch. cerevisiae Y185, and their presence in isolated plasma-membranes with low levels of [1-^{14}C]oleoyl residue activity, has indicated a possible role for low-density vesicles in transfer of incorporated exogenous fatty acids between triacylglycerol and phospholipid molecules at the plasma membrane. This may be supported by previous reports. Cartledge et al. (1977), although not showing plasma membrane/vesicle associations in their studies on low-density vesicles from Sacch. cerevisiae NCYC 366, proposed that phospholipid synthesis could occur at the plasma membrane, using diacylglycerols furnished by the low-density vesicles. They also thought it conceivable that hydrolysis of triacylglycerols to give diacylglycerols, catalyzed by a lipase in the low-density vesicles, occurs principally at the plasma membrane. Schaffner and Matile (1979) have also reported that low-density vesicles contain a lipase which hydrolyses triacylglycerols to diacylglycerols, the possible precursors of

phospholipids (Taylor and Parks, 1979). Studies with cultured neuroblastoma cells which incorporate exogenously supplied unsaturated fatty acids into their plasma membranes, have also provided evidence that phospholipid turnover in the plasma membrane can occur, at least in part, by a combination of deacylation-reacylation and de novo synthesis (Chakravarthy et al., 1986).

It would seem likely then, from the data presented in this thesis, that low-density vesicles of anaerobically grown Sacch. cerevisiae Y185 supplemented with oleic acid, while being effective reservoirs to accommodate an influx of fatty acids, may also play an important regulatory role in plasma-membrane phospholipid metabolism. Recently it has been shown that, in Dictyostelium discoideum (De Silva and Siu, 1981) and Acanthamoeba castellanii (Mills et al., 1984), phospholipid-rich intracellular vesicles are involved in transfer of phospholipids from the endoplasmic reticulum to the plasma membrane. Temperature-sensitive secretory mutants of Sacch. cerevisiae have been used to study the role of vesicles in the transfer of proteins from their site of synthesis to the plasma membrane (Schekman, 1982; Schekman and Novick, 1982). Daum et al. (1986) have used these mutants to establish whether the same vesicles are important in supplying the plasma membrane with phospholipids. While showing that other mechanisms were effective in supplying the plasma membranes of Sacch. cerevisiae with phospholipids, they could not exclude the possibility that vesicles might also serve as vehicles for phospholipid translocation. The data reported in this thesis are not indicative of low-density vesicle involvement in intracellular transfer of phospholipids to

plasma membranes, but rather show a role for low-density vesicles in phospholipid metabolism at the plasma-membrane itself.

The results on incorporation of oleic acid into anaerobically grown Sacch. cerevisiae Y185 have confirmed previous reports on vesicular associations with the plasma membrane. It would appear that low-density vesicles have a role as storage organelles for influx of essential fatty acids in anaerobically grown yeasts and, in some cases, the data reported here have shed more light on their involvement in plasma-membrane lipid metabolism. The observation made of the changes that occur during formation of spheroplasts was a particularly significant one. In previous reports, many workers have given some very accurate hypothetical suggestions as to the roles of low-density vesicles in plasma-membrane phospholipid synthesis during cell growth. However, without wishing to appear too sceptical, in light of the technical difficulties involved in obtaining subcellular organelles without altered lipid compositions, many of these workers have appeared to assume too much from their data. Most of them, for example, seemed to be oblivious to the fact that the lipid compositions of their isolated subcellular organelles were not necessarily the same as those found in the organisms at the time of harvesting and therefore, not indicative of the physiological state to which they were referring.

LONG-CHAIN ALCOHOL PRODUCTION BY YEASTS

Occurrence of Long-Chain Alcohols in Yeasts

The work presented in this thesis constitutes the most extensive study yet to appear on production of long-chain alcohols by yeasts. There have been scattered reports on the natural occurrence of long-chain alcohols in yeasts, but in most studies these organisms have been grown on n-alkanes and the alcohols are found esterified with fatty acids (Davidova et al., 1978; Muratov et al., 1979; Zalashko et al., 1979; Zalashko and Salokhina, 1982). It is interesting to note that the six yeast strains examined, with the ability to produce long-chain alcohols under self-induced anaerobic conditions, were shown to produce long-chain alcohols with the same chain-length profile, namely $C_{16} > C_{18} > C_{14}$. Traces of C_{12} alcohol were also found in some extracts, but were only detectable by combined GLC-mass spectrometry. Significantly, none of the yeasts examined synthesized unsaturated primary alcohols. Muratov et al. (1979) reported that wax esters from C. guilliermondii contained saturated long-chain alcohols, but in this organism the major chain lengths were C_{10}, C_{16} and C_{17}. This yeast, however, was grown on a mixture of n-alkanes. It is therefore likely that these alcohols were a product of alkane oxidation, thus reflecting the chain length, to a certain degree, of the carbon source (Britton, 1984; Ratledge, 1980). Davidova et al. (1978) reported briefly that glucose-grown cultures of C. tropicalis synthesized wax esters, although greater amounts were produced when this yeast was grown on n-octadecane.

In addition, several reports have appeared of yeast lipid fractions which, from their mobilities, could well be long-chain primary alcohols (Watanabe and Takakuwa, 1984; Zalashko et al., 1979), although they are frequently reported as being 'unidentified'. Watanabe and Takakuwa (1984) separated lipid extracts from glucose-grown Sacch. rouxii using a Chromarod of Iatroscan TH-10 (Iatron Laboratories, Inc., Japan). They reported the occurrence of the usual neutral lipids, sterols, free fatty acids, triacylglycerols and sterol esters, but also found an unidentifiable peak between free sterols and free fatty acids. Zalashko et al. (1979) also found an unidentifiable spot in a similar position when analysing lipid extracts of glucose-grown Rh. glutinis by TLC. Both groups of workers developed their chromatograms in solvent systems not dissimilar from those used in the present study for separating neutral lipids. Alternatively, these frequently unidentified lipid fractions may be members of the highly unsaturated isoprenoid alcohol series, such as those identified in the present study with mobilities similar to free long-chain primary alcohols.

Due to the greater amount of long-chain alcohols produced by C. albicans under self-induced anaerobic conditions, as compared with the other five yeast strains, further studies on long-chain alcohol production were confined to this organism. Long-chain alcohol production by C. albicans, grown under self-induced anaerobic conditions, was confined to the stationary phase of growth, suggesting that long-chain alcohols in this yeast are secondary metabolites. This contrasts with the production of long-chain

alcohols by micro-organisms grown on n-alkanes, where the alcohols are intermediates in carbon catabolism and are very much a part of primary metabolism (Boulton and Ratledge, 1984). However, the present report of long-chain alcohol production by C. albicans also conflicts with the findings of Davidova et al. (1978). They found wax esters in glucose-grown C. tropicalis harvested from exponential-phase cultures. There have, however, been a few reports of n-alkane production by yeasts (Barron and Hanahan, 1961; Guerzoni et al., 1985; Kovác̆ et al., 1967). It may be possible, therefore, that the alcohol component of the wax esters isolated by Davidova et al. (1978) may have resulted from terminal oxidation of endogenously synthesized n-alkanes. Naccarato et al. (1972, 1974) reported detection of long-chain alcohols in non-alkane grown Escherichia coli harvested from stationary-phase cultures. As with the long-chain alcohols detected in C. albicans, Naccarato et al. (1972) identified tetradecanol, hexadecanol and octadecanol as being the major primary alcohols. However, these workers did not describe the time-course of alcohol production. Other non-alkane grown bacteria such as Acinetobacter calcoaceticus (Fixter and Fewson, 1974; Fixter and McCormack, 1976; Fixter et al., 1986), harvested from stationary-phase cultures, and Micrococcus cryophilus (Lloyd and Russell, 1983a; Russell and Volkman, 1980), harvested from late exponential-phase cultures, have been studied for their wax-ester content. Again, a time-course for their production was not reported. The data presented in this thesis suggests that, in C. albicans, long-chain alcohols are secondary metabolites that are most probably produced by reduction of fatty acids.

No attempt was made in the present study to establish where long-chain alcohols are located in C. albicans. These lipids have been reported in the outer membranes of an Acinetobacter species (Thorne et al., 1973), but it has been assumed that these were probably derived from wax esters (Lloyd and Russell, 1983b). These latter workers found that most of the wax-ester content of the psychrophilic bacterium M. cryophilus is located in both the inner and outer membranes of the cell envelope. It is thought that they may have a function relating to regulation of membrane fluidity in this organism (Russell and Volkman, 1980). However, work on the effects of nutrient limitation on the wax-ester content of A. calcoaceticus (Fixter and Fewson, 1974; Fixter and McCormack, 1976; Fixter et al., 1986) has suggested that wax esters may be synthesized and used as energy reserves in this organism.

What I did, however, try to establish was whether and to what extent long-chain alcohols were present in C. albicans in the free and esterified form. Having discovered that lipid extracts of this organism contained wax esters, it seemed possible that these esters contained residues of long-chain alcohols which might be hydrolysed during extraction of lipids. Lipases are enzymes that hydrolyse ester bonds of water-immiscible substrates at an interface between the insoluble substrate phase and the aqueous phase in which the enzyme is soluble. As an interface is essential for a lipase to act, the presence of an organic solvent does not necessarily denature the enzyme (Ratledge and Evans, 1987). Therefore, certain yeast lipases may become activated during lipid extraction (Letters

and Snell, 1963). Crude lipase assays conducted in the present study provided evidence for lipase activity in C. albicans which was sensitive to pCMB, a lipase inhibitor (Schousboe, 1976). Inclusion of this inhibitor in the yeast culture, prior to harvesting, and during lipid extraction, although causing a considerable decrease in the content of free long-chain alcohols in extracts, did not lead to a corresponding increase in the size of the wax-ester fraction. It is likely, therefore, that long-chain alcohol residues are present in C. albicans in some other as yet unknown molecular form. Glycerol ether lipids, as well as wax esters, are widely distributed in nature (Harwood and Russell, 1984; Mahadevan, 1978). It may be possible that the long-chain alcohols of C. albicans are precursors of these lipids. As to whether these alcohols function as membrane lipids or storage lipids in stationary-phase cultures of this organism is uncertain. Due to the relatively low contents of these alcohols, it may be possible that they are simply a product of a futile metabolic pathway induced by cultural conditions.

Effect of Glucose Concentration on Long-Chain Alcohol Content and Fatty-Acyl Composition of Candida albicans

Due to the fact that two systems using media with different glucose concentrations were used for screening several yeast strains, one could not rule out the possibility that the difference in long-chain alcohol content observed with C. albicans, under aerobic and self-induced anaerobic conditions, could have been a function of glucose concentration. When C. albicans was grown

aerobically in medium similar in composition to that used for self-induced anaerobic cultures, long-chain alcohol production was still confined to the stationary phase of growth. Moreover, total alcohol yield was similar to that of organisms grown under self-induced anaerobic conditions. Final biomass yield, however, had approximately doubled.

Varying the glucose concentration in aerobic and self-induced anaerobic cultures of C. albicans had a marked effect on the long-chain alcohol content of the organism. Quite clearly, the effect of glucose concentration on long-chain alcohol production was different in aerobic as compared with self-induced anaerobic cultures. Not only was there a shift in the alcohol content of organisms with different glucose concentrations under aerobic and self-induced anaerobic conditions, but the profiles of the three major long-chain alcohols also changed. Synthesis of long-chain alcohols by C. albicans was clearly favoured by self-induced anaerobic conditions as well as high glucose concentrations, both of which are conditions which repress formation of mitochondria (Käppeli, 1986; Sols et al., 1971). Therefore, production of these long-chain alcohols might be explained by the repressive conditions diverting acyl-CoA derivatives of fatty acids to reductive routes. It has been shown that many Candida species (Einsele et al., 1972; Johnson et al., 1972; Skipton et al., 1974) have a repressed oxidative metabolism in the presence of high concentrations of glucose together with oxygen limitation. It is possible, then, that the shift observed in long-chain alcohol content with different glucose concentrations, between aerobic and self-induced anaerobic

conditions, could be explained by the fact that even at very high glucose concentrations (10%, w/v), aerobic conditions are still less repressive than the self-induced anaerobic conditions.

If long-chain alcohol production in C. albicans is a consequence of mitochondrial activity, the next, perhaps obvious, step was to examine the effect of other carbon-substrates on production of long-chain alcohols. The discovery that substituting galactose or glycerol for glucose in the medium greatly decreased or eliminated long-chain alcohol production suggests that alcohol synthesis is subject to carbon catabolite control. Galactose, while being another fermentable carbon source, is not as efficient as glucose at causing respiratory repression in C. albicans (Meyer et al., 1984; Odds, 1979). Brown and Johnson (1970) found that higher concentrations of galactose, as distinct from glucose, were required to induce fermentative activity in Sacch. cerevisiae. It would, therefore, seem not unreasonable to suggest that the inefficiency of galactose to induce mitochondrial repression could be responsible for the decreased alcohol content of C. albicans when grown on this substrate. Candida albicans is unable to use glycerol as a fermentable carbon source, but it is capable of respiring it (Meyer et al., 1984; Odds, 1979). The fact that this organism would have an active respiratory system when grown on glycerol may explain its inability to synthesize significant amounts of long-chain alcohol.

It would seem possible, then, that mitochondria are, in C. albicans, organelles which indirectly exert some type of control over synthesis of long-chain alcohols. However, the finding that

glucose concentration, in aerobic or self-induced anaerobic cultures, markedly affects alcohol content indicates that the regulatory effect of glucose on long-chain alcohol synthesis is complex. Synthesis of another secondary metabolite, a glycolipid, by the yeasts Torulopsis bombicola and T. magnoliae has been reported to be affected by the concentration of glucose in the growth medium (Spencer et al., 1979). Medium containing 10% (w/v) glucose generally gave better yields than media containing lower concentrations of glucose, although the mechanism of action is unknown. Martín (1979) has reported that macrolide antibiotic biosynthesis by Streptomyces fradiae is inhibited by high concentrations of glucose. This is believed to be due to its inhibitory effect on fatty-acid degradation, the products of which act as precursors for production of these secondary metabolites. Again, the molecular mechanism of this carbon catabolite regulation is not known. If high concentrations of glucose are responsible for regulating fatty-acid metabolism after cessation of growth, it is possible that, in C. albicans, long-chain alcohols arise by reduction of CoA esters of fatty acids that alternatively would be channelled into other cellular lipids.

It was considered that the different long-chain alcohol profiles observed in C. albicans, under aerobic and self-induced anaerobic conditions, may have reflected the fatty-acyl composition of the organism. Several workers have reported that long-chain alcohols found in wax esters isolated from Euglena gracilis (Inui et al., 1983), M. cryophilus (Lloyd and Russell, 1983a; Russell, 1978; Russell and Volkman, 1980) and A. calcoaceticus (Fixter and Fewson, 1974; Fixter and McCormack, 1976; Fixter et al., 1986) have

similar chain lengths to the fatty-acyl residues. The long-chain alcohols isolated from C. albicans and the other five yeast strains, in the present study, had similar chain lengths to the fatty-acyl residues reported for yeast lipids in general (Cottrell et al., 1986; Kaneko et al., 1976; Viljoen et al., 1986). The fatty-acyl compositions of C. albicans certainly reflected the conditions under which the organisms were grown. Under self-induced anaerobic conditions, as compared with aerobic conditions, there was an increased proportion of shorter-chain saturated fatty-acyl residues with a corresponding decrease in longer-chain unsaturated residues. This phenomenon has been reported by several other workers for yeast cultures grown under conditions of lowered oxygen tension (Ahvenainen, 1982; Babij et al., 1969; Jollow et al., 1968). The decrease in relative proportions of unsaturated fatty-acyl residues observed in aerobically grown C. albicans, when glucose concentrations were increased, is almost certainly due to the high concentrations of glucose leading to a situation more or less similar to that of self-induced anaerobic conditions (Lowden et al., 1972; Sols et al., 1971).

Despite the effects of cultural conditions on the relative proportions of fatty-acyl residues in C. albicans, there was no direct correlation between these and the proportions of long-chain alcohols. For example, the relatively high proportion of $C_{14:0}$ fatty-acyl residues in self-induced anaerobic cultures of C. albicans, containing 20% (w/v) glucose, was not necessarily the reason for the relatively high content of tetradecanol in this organism. This is borne out by the fact that the relative proportions of $C_{14:0}$ fatty-acyl residues in aerobically grown

C. albicans, when grown in medium containing 20% (w/v) glucose, was significantly lower, and yet the tetradecanol content was similar to that of organisms grown under self-induced anaerobic conditions. While availability of fatty-acyl residues is important for production of the respective long-chain alcohols, the proportions of these alcohols are more likely to be dependent on the fatty-acyl chain-length specificities of the enzymes involved in the reduction (Fixter et al., 1986). That none of the yeasts examined synthesized unsaturated alcohols and that C. albicans only produced trace amounts of dodecanol under self-induced anaerobic conditions, although there was a relatively high proportion of $C_{12:0}$ fatty-acyl residues under these conditions, would seem to suggest that the appropriate enzymes involved in reduction of fatty acids have a distinct fatty-acyl specificity. Inui et al. (1983) reported that the wax esters isolated from E. gracilis had different relative proportions of long-chain alcohol residues compared with those of the fatty acids. This was explained by the fact that the fatty acyl-CoA reductase in this organism was highly specific for myristoyl-CoA and palmitoyl-CoA (Kolattukudy, 1970). Many other organisms that produce alcohols by reduction of fatty acids have been shown to contain reductases with varying degrees of fatty-acyl specificity (Riendeau and Meighen, 1985).

Effect of Fatty Acid Supplementation on the Long-Chain Alcohol Content of Candida albicans

The discovery that C. albicans, when grown in media supplemented with odd chain-length saturated fatty acids,

synthesized long-chain alcohols of corresponding chain length is a particularly significant one. As shown by the fatty-acid analysis of organisms from unsupplemented cultures of C. albicans, odd chain-length fatty-acyl residues were not detected in any of the lipid classes. Many yeasts, including C. albicans, do not synthesize odd chain-length fatty-acyl residues unless grown on, or with, odd chain-length hydrocarbons (Rattray et al., 1975; Ratledge, 1980; Ratledge and Evans, 1987), which suggests that some of the odd chain-length fatty acids used in the present study were taken up by the organisms and reduced to the corresponding odd chain-length alcohols. Other yeasts, such as strains of Sacch. cerevisiae, are able to incorporate exogenously supplied fatty acids, residues of which they cannot synthesize, into their cellular lipids when grown under strict anaerobic conditions (Andreasen and Stier, 1954; Alterthum and Rose, 1973) or under the self-induced anaerobic conditions used in the present study (A. Rees, C.P. Cartwright and A.H. Rose, unpublished observations). In vivo studies with E. coli have indicated that exogenously provided fatty acids may serve as precursors of long-chain alcohols (Naccarato et al., 1974). These workers found that organisms from stationary-phase cultures, grown in the presence of [1-^{14}C] hexadecanoic acid, contained low levels of [1-^{14}C]hexadecanol; less than 0.2% of the incorporated acid was reduced to the alcohol.

The evidence that chain elongation and β-oxidation occur in C. albicans when $C_{13:0}$ and $C_{19:0}$ fatty-acid supplements, respectively, are used, would seem to suggest that these acids need to be modified before they are incorporated into cellular lipids. From

the nature of the odd-chain alcohols produced after cultures had been supplemented with $C_{15:0}$ and $C_{17:0}$ fatty acids, it would appear that very little modification of these acyl chains takes place. This could be explained by the fact that $C_{15:0}$ and $C_{17:0}$ acids are similar in chain length to the major fatty-acyl residues found in C. albicans (in this study) and other yeasts (Hunter and Rose, 1971; Ratledge and Evans, 1987). These types of modification have been observed in other yeasts. Tulloch et al. (1962) working with T. bombicola, found that supplementing cultures with C_{16} to C_{19} fatty acids led to their incorporation into glycolipids. Formation of odd chain-length hydroxy acids from odd chain-length substrates, which did not naturally occur in unsupplemented cultures, showed that the supplement was used directly without modification of chain length. However, when fatty acid supplements had chain lengths greater than C_{19}, Tulloch et al. (1962) reported evidence for β-oxidation. Although there is evidence for fatty-acid degradation in C. albicans, it is unlikely that the exogenously provided odd chain-length fatty acids would have contributed to synthesis of even chain-length alcohols. This would require α-oxidation of the substrate to form an aldehyde one carbon atom shorter (Fulco, 1967) followed by a reduction to the corresponding alcohol. If this system existed, odd-chain fatty acids and alcohols would be detectable in unsupplemented cultures of C. albicans.

Growing C. albicans in the presence of fatty acids probably resulted in a high proportion being incorporated into other cellular lipids, as has been observed with E. coli (Naccarato et al., 1974). Since C. albicans did not produce any more even

chain-length alcohols, when cultures were supplemented with even chain-length fatty acids, it can be assumed that this organism synthesizes sufficient endogenous even chain-length fatty acids for production of corresponding alcohols. The increased amount of heptadecanol, observed when cultures of C. albicans were supplemented every 24 h with heptadecanoic acid during the stationary phase of growth, was probably due to more of the incorporated acid being available for reduction. Supplementing cultures of T. bombicola with fatty-acid precursors, at 12 to 24 h intervals, as opposed to adding them all early on in the fermentation, has been reported to increase their incorporation into glycolipid (Spencer et al., 1979).

The decrease in production of hexadecanol and octadecanol by C. albicans, following increased availability of heptadecanoic acid, indicates that enzymes involved in the reduction of endogenous fatty acids are just as capable of using the exogenously provided odd chain-length acid as a substrate. The fact that glucose concentration had a similar effect on production of heptadecanol, as it did with the three major even chain-length alcohols, suggests that once the exogenously provided fatty acid substrate is incorporated into the organism, its conversion to long-chain alcohol is also subject to the same metabolic control as endogenously synthesized fatty acids. Tetradecanol production was unaffected by the presence of heptadecanoic acid which might imply that enzymes with shorter acyl chain-length specificities are involved in production of this alcohol. Whether these are acyl-CoA synthetases (Groot et al., 1976; Shimizu et al., 1980) or acyl-CoA

and aldehyde reductases (Riendeau and Meighen, 1985), or a combination of both, is uncertain.

Reduction of Palmitoyl-CoA Using Cell-Free Extracts of Candida albicans

Production of [1-^{14}C]hexadecanol from [1-^{14}C]palmitoyl-CoA using cell-free extracts of C. albicans is a definitive piece of evidence showing that a pathway exists for reduction of fatty acids to long-chain alcohols. This has been shown to be the major pathway for long-chain alcohol production in many organisms (Mahadevan, 1978; Riendeau and Meighen, 1985). The rate of production of hexadecanol by cell-free extracts of C. albicans, while being of similar magnitude to those found in some other organisms (Bishop and Hajra, 1981; Natarajan and Sastry, 1976), is between 50 and 100 times less than that found in E. gracilis (Khan and Kolattukudy, 1973; 1975) and gourami roe (Griffith et al., 1981). In these latter two organisms wax esters constitute a large proportion of the cellular lipid, which may explain the high rate of alcohol production by cell-free systems. It must be emphasized, however, that the data reported in this thesis were from a preliminary study and that the values obtained were not maximal rates of production under optimal assay conditions. From the results obtained, it is obvious that most of the enzyme activity is located in the soluble protein fraction as opposed to the microsomal fraction. In general, the cell-free extracts that catalyze reduction of fatty acids to long-chain alcohols are either microsomal or soluble protein fractions (Riendeau and Meighen, 1985).

The observation that cell-free extracts of C. albicans catalyzed reduction of palmitoyl-CoA at the same rate when either NADH or NADPH was used as the coenzyme contrasts with other reports (Riendeau and Meighen, 1985). It has generally been found that microsomal acyl-CoA reductases are specific for NADPH, E. gracilis being the only exception, and the soluble systems NADH-specific. The alkane oxidase system from C. rugosa is capable of using NADH or NADPH as coenzymes (Boulton and Ratledge, 1984). Therefore, it may be possible that, in C. albicans, the reductases involved in conversion of fatty acids to long-chain alcohols are non-specific for NADH or NADPH. Alternatively, the crude enzyme preparations may have contained a transhydrogenase capable of interconverting NADH and NADPH (Gallo et al., 1973).

During this preliminary study, no attempt was made to isolate long-chain aldehydes. These lipids have been detected as intermediates in the cell-free conversion of fatty acids to long-chain alcohols by many organisms (Riendeau and Meighen, 1985). Furthermore, for some of the soluble fatty-acid and acyl-CoA reductases that have been partially purified from different sources, only the long-chain aldehyde was obtained as the reaction product (Day et al., 1978; Riendeau and Meighen, 1981). However, now that a cell-free conversion of palmitoyl-CoA to hexadecanol, mainly by soluble proteins, has been demonstrated in C. albicans, it will be possible to study further the enzymes that catalyze this conversion and the effect of glucose concentration on their synthesis and activity.

Are Yeasts a Viable Source of Long-Chain Alcohol?

Due to the pathogenicity of C. albicans to man (Odds, 1979), its use on an industrial scale is likely to cause many political problems. Other yeasts in this study, however, have been shown to produce long-chain alcohols. Since cultural conditions have a regulatory effect on alcohol production in C. albicans, it is likely that other yeasts, including some of those screened in this study, may be capable of producing significant amounts of long-chain alcohol. Due to the multistep nature of the bioconversion of fatty acids to long-chain alcohols however, and the necessity to activate the fatty acid and provide reducing equivalents (in the form of NADH or NADPH), operation of a viable commercial process using isolated enzymes is not feasible using existing technology (Drozd, 1980). In C. albicans, production of long-chain alcohols from either endogenously synthesized or exogenously provided fatty acids, while reaching more than trace amounts, is far from attaining acceptable commercial levels. If a whole-cell process is to be a successful alternative to isolated-enzyme systems, it will be necessary to use a yeast which will overproduce long-chain alcohols from fatty acids and, preferably, excrete them into the medium.

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Production of Long-chain Alcohols by Yeasts

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Fourteen yeast strains from six genera were analysed for the presence of long-chain alcohols. Six strains from three genera contained long-chain alcohols, highest levels being found in *Candida albicans*. The alcohols were identified and determined by TLC, GLC and GLC–MS. The major long-chain alcohols synthesized by these organisms were saturated, primary alcohols with C_{14}, C_{16} or C_{18} chain length. Unsaturated long-chain alcohols were not detected. In all strains that produced long-chain alcohols, the relative proportions were $C_{16} > C_{18} > C_{14}$. Long-chain alcohol contents were higher in organisms from anaerobically, as compared with aerobically, grown cultures reaching about 650 μg (g dry wt organisms)$^{-1}$ in stationary-phase cultures of *C. albicans*. In cultures of *C. albicans*, synthesis of long-chain alcohols occurred only after the end of exponential growth. The alcohols were predominantly present as free alcohols. The fatty-acyl chain-length profile of the triacylglycerol and to a lesser extent the sterol/wax ester fractions from *C. albicans* reflected that of the long-chain alcohols produced by this yeast.

INTRODUCTION

Long-chain alcohols occur as components of animal, higher plant and microbial lipids as free alcohols, precursors of glycerol ether lipids and in combination with carboxylic acids as wax esters (Mahadevan, 1978; Harwood & Russell, 1984). Among micro-organisms that synthesize long-chain alcohols, bacteria have been studied most intensively (Stewart & Kallio, 1959; Raymond & Davis, 1960; Baptist *et al.*, 1963; Day *et al.*, 1970; Allen *et al.*, 1971; Naccarato *et al.*, 1972; Lloyd & Russell, 1983). Some long-chain alcohol-containing bacteria, such as mycobacteria (Ratledge, 1976), have been studied because of their medical importance. Other bacteria have been investigated for their industrial importance in, for example, degradation of oil spillage (Higgins & Gilbert, 1978). In oil-degrading micro-organisms, long-chain alcohols are produced as intermediates in terminal oxidation of alkanes, and may also be found esterified with long-chain carboxylic acids which are produced by further oxidation of the alcohols (Ratledge, 1978; Britton, 1984). When grown on carbon sources other than alkanes, some bacteria synthesize long-chain primary alcohols by reduction of CoA esters of long-chain carboxylic acids which arise either from activity of the fatty acid synthase (Day *et al.*, 1970; Naccarato *et al.*, 1972; Lloyd & Russell, 1983) or are supplied exogenously (Naccarato *et al.*, 1974; Lloyd & Russell, 1983).

Some yeasts are able to grow on alkanes (Britton, 1984) and certain of these organisms may accumulate wax esters containing long-chain alcohols. These yeasts include *Candida guilliermondii* (Muratov *et al.*, 1979) and *Rhodotorula glutinis* (Zalashko *et al.*, 1979; Zalashko & Salokhina, 1982). Reports of long-chain alcohol production by yeasts grown on non-alkane

Abbreviations: PCMB, *p*-chloromercuribenzoate; TMS ethers, trimethylsilyl ethers.

substrates are rare. Davidova *et al.* (1978) reported that wax esters were detectable in glucose-grown *Candida tropicalis* harvested from exponential-phase cultures, although their synthesis was more prolific when this yeast was grown on alkanes. The present paper reports synthesis of long-chain alcohols by several yeasts in media containing glucose as the carbon source.

METHODS

Organisms. The yeasts used were *Candida albicans* NCYC 1467, *C. bombicola* NCYC 1449, *C. maltosa* Colworth Microbial Culture Collection (CMCC) 3152, *C. ingens* NCYC 822, *C. utilis* NCYC 168, *C. utilis* NCYC 707, *Debaryomyces hansenii* NCYC 9, *Pichia fermentans* NCYC 850, *Rhodotorula glutinis* NCYC 59, *Rh. glutinis* CMCC 2272, *Rh. rubra* NCYC 195, *Rhodotorula* sp. ATCC 20254, *Saccharomyces cerevisiae* Y 185 (a gift from J. R. Woodward, Department of Biotechnology, University of Leeds, UK) and *Saccharomycopsis* (*Yarrowia*) *lipolytica* ATCC 20225. They were maintained at 4 °C on slopes of malt extract–yeast extract–glucose–mycological peptone (MYGP) agar (Wickerham, 1951).

Experimental cultures. Organisms were grown aerobically in a medium containing (l^{-1}): glucose, 20 g; $(NH_4)_2SO_4$, 3·0 g; KH_2PO_4, 4·5 g; yeast extract (Lab M), 1·0 g; $MgSO_4.7H_2O$, 25 mg; and $CaCl_2.2H_2O$, 25 mg (adjusted to pH 4·5 with HCl). Growth under self-induced anaerobic conditions used the same medium except that the concentration of glucose was 200 g l^{-1} and that of yeast extract 4 g l^{-1}. One-litre portions of medium were dispensed into 2 l round flat-bottomed flasks and sterilized by autoclaving at $6{\cdot}89 \times 10^4$ Pa for 1 min. Flasks used for aerobic growth were plugged with cotton wool, and those used for self-induced anaerobic growth were fitted with fermentation locks (Beavan *et al.*, 1982). Starter cultures (100 ml medium in a 250 ml flask) were inoculated with a pinhead of organisms from a slant culture, and incubated for 48 h at 30 °C on an orbital shaker (200 r.p.m.). Experimental cultures used in the survey of long-chain alcohol production were inoculated with 1 ml of starter culture; those used for further studies were inoculated with a portion of starter culture containing 10 mg dry wt organisms. Cultures were incubated aerobically as described by Patching & Rose (1969) and under self-induced anaerobic conditions as described by Beavan *et al.* (1982). The latter cultures are described as anaerobic in this paper. Growth was followed by measuring optical density at 600 nm; measurements were related to the dry wt of each yeast strain using an appropriate standard curve. At the times indicated, organisms were harvested by centrifugation at 6000 *g* at 4 °C and washed twice with water.

Lipid extraction. Before harvesting, 2 ml solution containing 10 mg each of cycloheximide and chloramphenicol, and where indicated 10 ml 50 mM-PCMB in methanol, were injected into the culture which was incubated for a further 15 min. Lipids were extracted from washed organisms by a modification of the Folch *et al.* (1957) procedure. Washed organisms were resuspended in 20 ml methanol and shaken in a Braun homogenizer for four periods of 30 s at speed 2 (4000 r.p.m.) with glass beads (40 g; Sigma type V; 0·45–0·50 mm diam.). The sample bottle was cooled with expanding CO_2 during homogenization. Chloroform was added to the suspension to give 2:1 (v/v) chloroform/methanol, and the mixture stirred magnetically for 2 h at room temperature (18–22 °C). The suspension was then filtered through Whatman no. 44 filter paper and the extraction procedure repeated on the residue. Extracts were pooled, washed with 0·25 vol. 0·88% (w/v) KCl and the mixture left to separate overnight at −20 °C. The lower organic phase was removed, taken to dryness using a rotary evaporator, and the residue immediately dissolved in 1 ml light petroleum (b.p. 60–80 °C). Samples were stored under nitrogen gas at −20 °C. Where indicated the extraction suspension was supplemented with 1·67 mM-PCMB.

Analysis of free and esterified long-chain alcohols. Extracts were fractionated into lipid classes by chromatography on silicic acid columns prepared by a modification of the Naccarato *et al.* (1972) procedure. Graduated glass pipettes (E-MIL; 5 ml) were plugged with glass wool and filled with 1 g SIL-LC silicic acid (325 mesh; lipid chromatography grade) (Hirsch & Ahrens, 1958). Maximum suction from a water aspirator, applied to the lower end of the column, caused slight compaction of the contents and assured even and reproducible packing. Packed columns were saturated with 20 ml light petroleum by overpressure with high-purity nitrogen gas. Lipid extracts were applied to the top of the column and eluted with 10 ml each of 4%, 7% and 10% (v/v) diethyl ether in light petroleum. A flow rate of 0·5 ml min^{-1} was maintained by nitrogen pressure on the column, and eluates were collected in 3 ml fractions. Fractions were taken to dryness under a stream of nitrogen gas, redissolved in an appropriate volume of light petroleum and 20 µl portions spotted onto a 20 cm × 20 cm × 0·25 mm Silica Gel 60 precoated TLC plate (Merck). The plate was developed with a light petroleum (b.p. 40–60 °C)/diethyl ether/acetic acid (70:30:1, by vol.) solvent mixture (Mangold, 1969); lipids were located by spraying with 0·2% (w/v) 2′,7′-dichlorofluorescein in ethanol (Griffith *et al.*, 1981) and viewed under ultraviolet (254 nm) radiation. Standards [1 mg ml^{-1} light petroleum (b.p. 60–80 °C)] were cholesterol palmitate, tripalmitin, stearic acid, palmitic acid, hexadecanol, octadecanol and ergosterol. The method gave excellent separation of lipid classes from extracts (Fig. 1). Subsequent analysis of free long-chain alcohols used only fractions 6–9 from the silicic acid column (Fig. 1). To assay long-chain alcohols, appropriate fractions from the silicic acid column were pooled, concentrated under nitrogen gas and further separated by TLC using a solvent mixture of hexane/diethyl ether/acetic acid (30:70:1,

by vol.) (Naccarato *et al.*, 1972). Long-chain alcohols were visualized as described above. The appropriate areas of the plate were scraped off and lipids extracted using light petroleum/methanol/30% (w/v) NaCl (1:1:1, by vol.). Samples were shaken vigorously and allowed to separate. The top layer was removed and the extraction procedure repeated twice. Extracts were pooled and taken to dryness under nitrogen gas.

Long-chain alcohols were separated and identified by GLC after conversion to their trimethylsilyl (TMS) ethers. Samples were taken up in 0·5 ml pyridine, and the solution was mixed with an equal volume of bis(trimethylsilyl)trifluoroacetamide. The mixture was transferred to a 1 ml screw-top vial and heated at 70 °C for 15 min. Silylated samples were separated using a Pye Unicam PU 4500 gas chromatograph fitted with a 25 m SE30 capillary column. The injection temperature was 300 °C, and the detector temperature 350 °C. The initial column temperature was 190 °C, and this was increased after 25 min at a rate of 16 °C min^{-1} to give a final temperature of 250 °C which was maintained for a further 10 min. The carrier gas (helium) flow rate was 1 ml min^{-1}, and the nitrogen flow rate, as a make-up gas across the detector, was 40 ml min^{-1}. TMS ethers of long-chain alcohols were identified by comparing their retention times with those of known standards and by co-chromatography with authentic standards. Peaks were analysed using a Pye Unicam CDPI computing integrator, and quantified by reference to a 1-heptadecanol internal standard added during the extraction procedure after disruption of organisms. Identification of long-chain alcohols was verified by GLC–MS using electron-impact ionization or chemical ionization. Silylated long-chain alcohols and carboxylic acids were used, and typical outputs of total ion current versus time matched the GLC traces obtained with the Pye Unicam PU 4500 gas chromatograph described above. The instrument used was a VG Analytical 70/70E, with a DB1 capillary column programmed at 150 °C for 5 min and rising to 300 °C at 30 °C min^{-1} for 5 min and held at the upper temperature. The flow rate of the carrier gas (helium) was 1 ml min^{-1}, the resolution 1000, the electron impact 70 eV and the calibrated range 20–580.

To examine the effect of PCMB on extraction of esterified long-chain alcohols, fractions 1–3 from the silicic acid column, which contained neutral lipids, were pooled and applied to Silica Gel 60 plates. Lipids were separated using the solvent system light petroleum–diethyl ether–acetic acid (90:10:1, by vol.) (Kates, 1972), and visualized as described above. Lipid bands with mobilities similar to heptadecanyl acetate, the internal standard, were scraped off the plate, and the lipids extracted with light petroleum as described above. Samples were then dried under a stream of nitrogen gas, and saponified using a modification of the method of Pollard *et al.* (1979) by adding 5 ml 1 M-KOH in 95% (v/v) methanol, sealing and heating at 80 °C for 3 h. After cooling, samples were diluted with 5 ml methanol, and non-saponifiable lipids removed with 3 × 5 ml light petroleum. They were then prepared for GLC and analysed as described above.

Analysis of the fatty-acyl composition of lipids. Lipid extracts from organisms were dissolved in chloroform to give approximately 25 mg lipid ml^{-1}. To prepare fatty-acid methyl esters of total lipids from organisms, 1 ml lipid solution was taken to dryness under a stream of nitrogen gas. BF_3 (14%, w/v, in methanol; 3 ml) was added and the mixture was heated for 1 h at 80 °C in a sealed vial. To extract fatty-acid methyl esters, the reaction mixture was made up to 5 ml with methanol and supplemented with 5 ml each of light petroleum and 30% (w/v) NaCl. The solution was shaken vigorously and the mixture left to separate, the procedure being repeated twice more. The solution of fatty-acid methyl esters was taken down to a small volume under a stream of nitrogen gas, streaked onto a 20 cm × 20 cm × 0·25 mm Silica Gel 60 precoated TLC plate (Merck) and the plate developed with a light petroleum/diethyl ether/formic acid (75:25:0·5, by vol.) solvent mixture (Fixter *et al.*, 1986). Methyl esters were scraped off the plate, extracted with light petroleum as described above, and taken down to a small volume under a stream of nitrogen gas. Fatty-acid methyl esters were analysed using a fused silica capillary column (25 m length; SGE BP21) in a Pye Unicam GCD chromatograph fitted with an SGE on-column adaptor. The injection temperature was 240 °C and the detector temperature 280 °C. The column was maintained at 135 °C for the first 5 min, after which the temperature was raised at the rate of 8 °C min^{-1} until it reached 180 °C. The carrier gas was hydrogen. Percentage fatty-acyl compositions were calculated using a Pye Unicam CDPI computing integrator. Individual lipid classes in total lipid extracts were separated by streaking a solution containing 10 mg lipid onto a Silica Gel 60 precoated TLC plate as above, which was developed with a solvent mixture containing light petroleum (b.p. 40–60 °C)/diethyl ether/acetic acid (70:30:1, by vol.). Lipid classes were identified as described above, bands scraped off the plates, and fatty-acid methyl esters of the lipids prepared as described above.

Chemicals. All chemicals used were AnalaR or of the highest purity available.

RESULTS

Identification of long-chain alcohols

The mobility of certain lipid fractions on silicic acid columns and on TLC (Fig. 1) suggested that their polarity was that expected of long-chain alcohols. Subsequently purification,

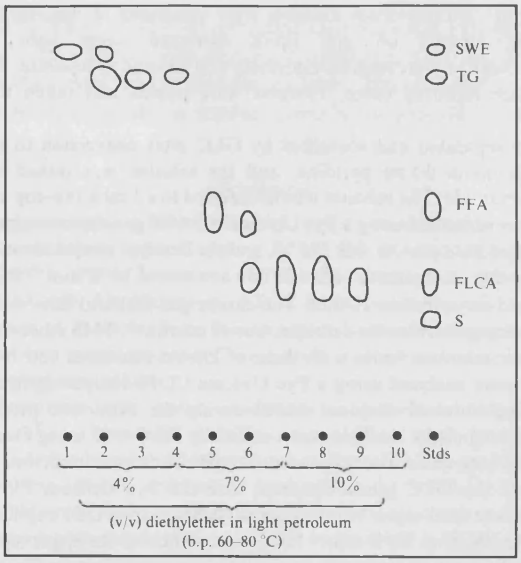

Fig. 1. Drawing of a TLC plate showing separation of lipid classes in each of the ten 3 ml fractions obtained by silicic acid chromatography of a lipid extract of *C. albicans* harvested from cultures after 72 h growth under anaerobic conditions. SWE, Sterol/wax esters; TG, triacylglycerols; FFA, free fatty acids; FLCA, free long-chain alcohols; S, sterols; Stds, lipid standards.

separation and identification by GLC and GLC–MS confirmed the presence of C_{14}, C_{16} and C_{18} primary alcohols in extracts of organisms. Fig. 2(*a*) shows the total ion-current trace from GLC–MS of a lipid extract from anaerobically grown *C. albicans* after purification by TLC. The peak at scan no. 253 was identified as 1-hexadecanol from its fragmentation pattern in electron-impact ionization MS (Fig. 2*b*). Peaks 36, 115 and 362 were generated by C_{12}, C_{14} and C_{18} long-chain alcohols, respectively. Peak 309 was due to the 1-heptadecanol internal standard. Other components of the extract identified were members of the isoprenoid alcohol series (peaks 111, 128, 378 and 403). This latter group of compounds was found in all lipid extracts examined by TLC, and they were also identified in extracts of yeasts that lacked the ability to synthesize long-chain alcohols.

Survey of long-chain alcohol production by yeasts

A survey was made of the ability of 14 strains of yeast to produce long-chain alcohols. Strains were selected partly on the basis of their known ability to metabolize exogenously provided lipids. All strains grew under aerobic conditions although five were unable to do so under anaerobic conditions (Table 1). While some of the strains produced saturated long-chain alcohols, none produced unsaturated long-chain alcohols. Only three yeasts, *C. albicans*, *C. utilis* NCYC 168 and *P. fermentans*, accumulated appreciable amounts of long-chain alcohols when grown either anaerobically or aerobically (Table 1). *C. maltosa*, *C. utilis* NCYC 707 and *S. cerevisiae* contained these alcohols when grown anaerobically, but little or no such alcohol after aerobic growth. In general, anaerobic conditions favoured production of long-chain alcohols. Six of the strains with ability to produce the alcohols under these conditions were examined quantitatively for content of C_{14}, C_{16} and C_{18} alcohols (Table 2). The content of C_{16} alcohols was greatest after anaerobic growth with all of the strains examined. In *C. albicans* grown under aerobic conditions, C_{18} alcohol was found in slight excess over C_{16} alcohol, although the total long-chain alcohol content was about fourfold greater in organisms from anaerobically grown cultures of this yeast. Production of all alcohols, especially C_{16}, was greatest by *C. albicans* grown under anaerobic conditions.

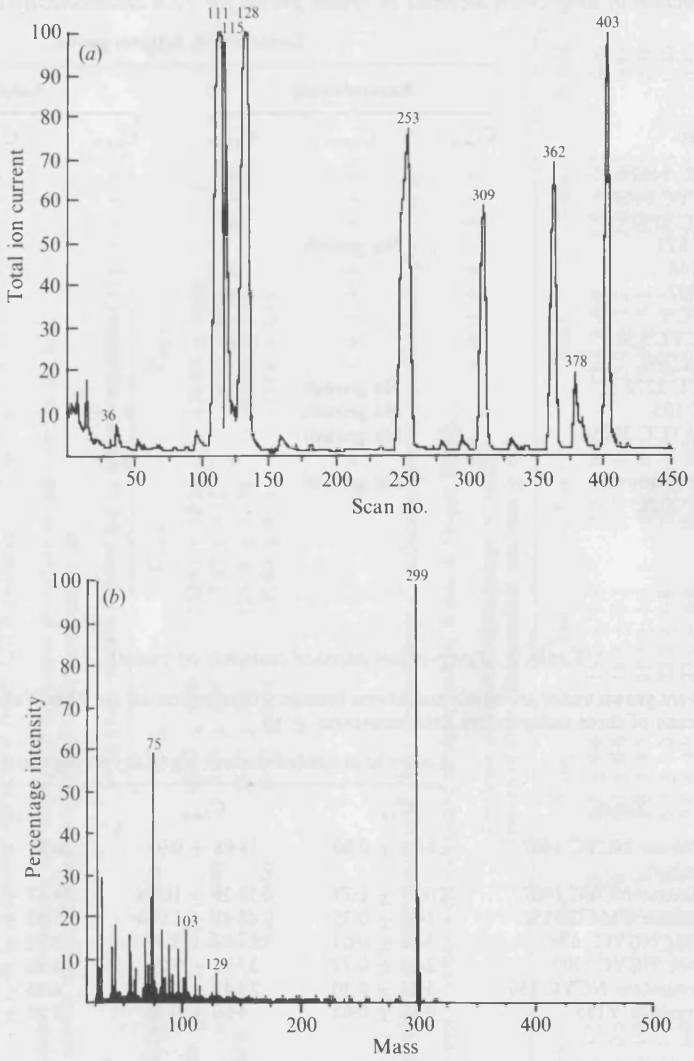

Fig. 2. (*a*) Total ion-current trace of long-chain alcohol TMS ethers in GLC–MS. Numbers against peaks are scan numbers. (*b*) Electron-impact ionization mass spectrum of peak scan no. 253 of (*a*). This peak was recognized as having been generated by hexadecanol. Long-chain alcohols were purified using extracts of *C. albicans* harvested after 168 h growth under anaerobic conditions.

Long-chain alcohol production by C. albicans under anaerobic conditions

Further studies were confined to *C. albicans* grown under anaerobic conditions. The yeast had a doubling time of 110 min, and reached stationary phase after 48 h (Fig. 3). Only traces of long-chain alcohols were detected in organisms from exponentially growing cultures. However, after cultures entered stationary phase, the contents of C_{14}, C_{16}, and C_{18} alcohols increased rapidly until about 170 h after which alcohol contents remained constant. Rates of increase in alcohol content during stationary phase were greatest for C_{16} alcohols and smallest for C_{14} alcohols (Fig. 3). Long-chain alcohols were not detected in culture filtrates except after prolonged (500 h) incubation, when the low concentrations present were probably due to limited lysis of organisms.

Table 1. *Detection of long-chain alcohols in yeasts grown for 72 h anaerobically or aerobically*

Yeast	Detection in cultures grown: Anaerobically $C_{14:0}$	$C_{16:0}$	$C_{18:0}$	Aerobically $C_{14:0}$	$C_{16:0}$	$C_{18:0}$
C. albicans NCYC 1467	+	+	+	+	+	+
C. bombicola NCYC 1449	−	−	−	−	−	−
C. maltosa CMCC 3152	+	+	+	−	−	−
C. ingens NCYC 822		No growth		−	−	−
C. utilis NCYC 168	+	+	+	+	+	+
C. utilis NCYC 707	+	+	+	−	−	−
D. hansenii NCYC 9	−	−	−	−	−	−
P. fermentans NCYC 850	+	+	+	+	+	+
Rh. glutinis NCYC 59	−	−	−	−	−	−
Rh. glutinis CMCC 2272		No growth		−	−	−
Rh. rubra NCYC 195		No growth		+/−	+/−	+/−
Rhodotorula sp. ATCC 20254		No growth		−	−	−
S. cerevisiae Y185	+	+	+	+/−	+/−	+/−
Saccharomycopsis (*Yarrowia*) *lipolytica* ATCC 20225		No growth		−	−	−

Table 2. *Long-chain alcohol contents of yeasts*

Cultures were grown under anaerobic conditions (except where indicated) for 72 h. Values quoted are the means of three independent determinations ± SD.

Yeast	Long-chain alcohol content [μg (g dry wt organisms)$^{-1}$] $C_{14:0}$	$C_{16:0}$	$C_{18:0}$
C. albicans NCYC 1467 (aerobic)	8·76 ± 0·80	18·08 ± 0·97	26·31 ± 2·03
C. albicans NCYC 1467	20·59 ± 1·79	139·29 ± 10·24	34·47 ± 2·82
C. maltosa CMCC 3152	1·76 ± 0·35	47·91 ± 13·84	5·62 ± 1·01
C. utilis NCYC 168	3·30 ± 0·63	17·93 ± 3·47	3·92 ± 0·82
C. utilis NCYC 707	2·13 ± 0·72	27·05 ± 9·26	4·42 ± 0·98
P. fermentans NCYC 850	3·25 ± 0·30	28·47 ± 7·88	4·38 ± 0·68
S. cerevisiae Y185	0·89 ± 0·02	4·66 ± 0·48	1·35 ± 0·55

Effect of PCMB on extraction of free and esterified long-chain alcohols from C. albicans

Supplementing cultures with the lipase inhibitor PCMB (Schousboe, 1976) just before harvesting, and including this compound during the extraction procedure up to the stage at which filtered chloroform/methanol extracts were pooled, appreciably decreased the apparent content of all three alcohols (Table 3). The possibility that a proportion of the alcohols was esterified was therefore examined using unsupplemented extractions and extractions containing PCMB. Only a small proportion was found to be esterified in this fraction (Table 3).

Fatty-acyl composition of total lipids and lipid classes in C. albicans

The major fatty-acyl residues in all of the fractions analysed (Table 4) were C_{16} and C_{18}, including both saturated and unsaturated residues. In the triacylglycerol fractions and in the total lipid extract, the proportion of $C_{14:0}$ residues was also high, much more so than in the phospholipid, sterol/wax ester and free fatty-acid fractions.

Table 3. *Effect of PCMB supplementation on contents of free and esterified long-chain alcohols in C. albicans harvested from 120 h cultures*

PCMB was included in the culture and during the extraction procedure as indicated in the text. Values quoted are the means of three independent determinations ± SD.

Inhibitor	Fraction	Long-chain alcohol content [μg (g dry wt organisms)$^{-1}$]		
		$C_{14:0}$	$C_{16:0}$	$C_{18:0}$
None	Free	90·57 ± 5·69	252·91 ± 10·50	135·65 ± 14·86
	Wax ester	8·23 ± 0·64	7·47 ± 0·12	6·54 ± 0·16
PCMB	Free	55·77 ± 3·0	125·96 ± 16·79	61·08 ± 3·86
	Wax ester	19·47 ± 0·54	22·85 ± 0·71	12·08 ± 0·61

Table 4. *Fatty-acyl composition of total lipids and lipid classes from C. albicans grown anaerobically for 168 h*

Δ mol^{-1} values were calculated as described by Kates & Hagen (1964).

Lipid	Contents of residues (percentage of total) (means of three independent analyses ± SD)								
	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	Δ mol^{-1}
Total	11·3 ± 1·3	18·4 ± 1·2	25·9 ± 1·3	6·7 ± 0·6	15·2 ± 1·8	7·8 ± 0·1	13·3 ± 0·9	1·7 ± 0·2	0·46
Phospholipids	1·5 ± 0·2	4·3 ± 0·5	25·4 ± 1·0	6·3 ± 0·6	18·3 ± 0·3	16·2 ± 1·8	25·2 ± 1·2	2·8 ± 0·1	0·81
Triacylglycerols	11·6 ± 1·1	20·6 ± 1·9	22·8 ± 0·8	5·3 ± 0·5	21·3 ± 1·6	7·1 ± 0·6	9·9 ± 0·3	1·3 ± 0·1	0·36
Sterol/wax esters	14·5 ± 1·7	13·3 ± 0·5	23·5 ± 1·6	6·3 ± 1·5	22·2 ± 0·8	8·7 ± 1·1	10·2 ± 1·2	1·4 ± 0·3	0·40
Free fatty acids	7·3 ± 2·0	7·4 ± 0·9	24·3 ± 0·7	7·4 ± 3·9	27·1 ± 6·0	10·3 ± 1·0	14·2 ± 0·9	2·1 ± 0·5	0·52

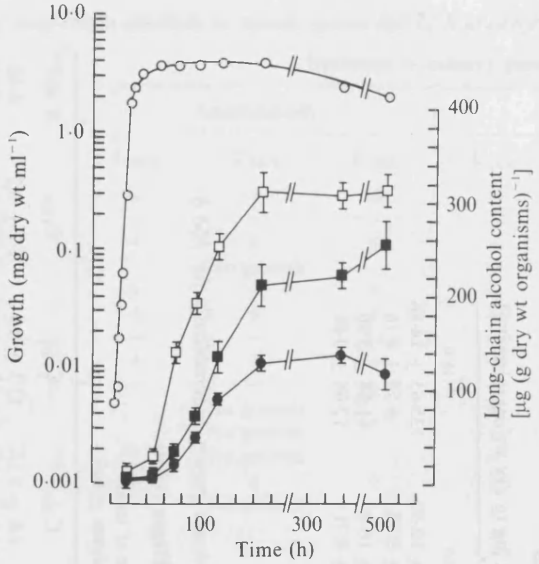

Fig. 3. Time-course of growth (○) of *C. albicans* and of its contents of $C_{14:0}$ (●), $C_{16:0}$ (□) and $C_{18:0}$ (■) long-chain alcohols. Values quoted are the means of three independent analyses ±SD.

DISCUSSION

The present paper is the most extensive study published on long-chain alcohols in yeasts. Davidova *et al.* (1978) reported briefly that glucose-grown *C. tropicalis* synthesizes wax esters. In addition, several reports have appeared of yeast lipid fractions which, from their mobilities, could well be long-chain alcohols (Zalashko *et al.*, 1979; Watanabe & Takakuwa, 1984), although they are frequently reported as being 'unidentified'. The present report of long-chain alcohol production by *C. albicans* contrasts with that by Davidova *et al.* (1978) in that alcohol production was confined to the stationary phase of growth, suggesting that long-chain alcohols in this yeast are secondary metabolites. Naccarato *et al.* (1972, 1974) reported detection of long-chain alcohols in *Escherichia coli* harvested from stationary-phase cultures but they did not describe the time-course of alcohol production.

All of the yeasts which in the present study were shown to produce long-chain alcohols synthesized these compounds with the same chain-length profile, namely $C_{16} > C_{18} > C_{14}$. Traces of C_{12} alcohol were also detected in some extracts. Significantly, none of the yeasts examined synthesized unsaturated alcohols. The chain-length profile is similar to the fatty-acyl residue profile reported for yeasts in general (Rattray *et al.*, 1975; Kaneko *et al.*, 1976). The fatty-acyl composition of total lipid extracts of *C. albicans*, and specifically of the triacylglycerol and sterol/wax ester fractions, was particularly rich in C_{14} residues, much more so than most other yeasts. It is tempting to suggest that saturated long-chain alcohols arise by reduction of CoA esters of long-chain carboxylic acids that alternatively would be channelled into other neutral lipids, a route which has been demonstrated in a variety of other organisms (Riendeau & Meighen, 1985). The switch from incorporation into triacylglycerols and other neutral lipids into long-chain alcohols would appear to be associated with cessation of growth. Synthesis by reduction of long-chain carboxylic acids is the more likely route than α-oxidation to the corresponding aldehyde (Fulco, 1967) followed by reduction to the alcohol, since the latter route would yield predominantly odd-chain alcohols which were not detected in any of the yeasts studied.

Synthesis of long-chain alcohols by the yeasts studied was clearly favoured by anaerobic conditions which repress formation of mitochondria (Käppeli, 1986). This might be explained

by the anaerobic conditions diverting acyl-CoA derivatives to reductive routes. Repression of mitochondrial synthesis would have been increased by the rather high concentration of glucose in the medium used for aerobic and particularly anaerobic growth. Possibly, therefore, mitochondria are, in *C. albicans*, organelles which indirectly exert some type of control over synthesis of long-chain alcohols.

No attempt was made in the present study to establish where long-chain alcohols are located in *C. albicans*. We did, however, try to establish whether and to what extent these alcohols were present in organisms in the free and esterified form. Having discovered that lipid extracts of *C. albicans* contained wax esters, it seemed possible that these esters contained residues of long-chain alcohols which might be hydrolysed during extraction of lipids from the yeast. Inclusion of the lipase inhibitor PCMB in the yeast culture and during lipid extraction, although causing a considerable decrease in the content of long-chain alcohols in extracts, did not lead to a corresponding increase in the size of the wax-ester fraction. It is likely, therefore, that long-chain alcohol residues are present in *C. albicans* in some other as yet unknown molecular form.

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